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PROCEEDINGS OF THE ROYAL SOCIETY.

SECTION B.—BIOLOGICAL SCIENCES.

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Studies on Epithelial Phagocytosis.—I.

By H. M. CARLETON, M.A., B.Sc., D.Phil., Department of Physiology, and
Sir William Dunn School of Pathology, Oxford.

(Communicated by Sir Charles Sherrington, F.R.S.—Received November 6, 1930.)

[PLATES 1, 2.]

I. *Introduction.*

The observations here described arose out of a research (now in the press) made by Florey and myself for the Birth Control Investigation Committee. Wishing to ascertain whether aspiration was exerted by the uterus during coitus, we injected animals *per vaginam* with suspensions of carmine or graphite immediately before copulation. When examining sections of the vaginae later, I was struck by the apparent inclusion of particles of the injection mass in the vaginal epithelial cells. Subsequent study has demonstrated that the epithelium of the upper segment of the rabbit's vagina is definitely phagocytic, as the experimental evidence detailed below will show.

II. *Material and Methods.*

The present study is largely based upon a series of 40 rabbits. Two methods of experimental approach were used.

The *first* and simplest consists of injecting the suspension *per vaginam*. Passage of a fine gum-elastic catheter has, however, disadvantages. Considerable resistance may be offered, nor does the administration of ether always abolish this spasm. Trauma, moreover, is easily elicited, even with the most

careful manipulation. The best method is to inject a large volume of suspension through a very short length of catheter attached to a syringe; in this manner any possibility of damage to the upper segment of the vagina is avoided.

The *second* method consists in introducing the material directly into the upper end of one uterine horn. It is possible in this way to test the phagocytic power of the uterine as well as the vaginal mucosæ. Substances injected into the uterus are rapidly passed down it (presumably by peristalsis) into the vagina. Injection of the *ora uteri* under direct vision is, of course, impossible. The uterine horns, therefore, are exposed through a median laparotomy wound under ether anæsthesia, the other abdominal viscera being packed off by gauze sponges wrung out of hot saline. One uterine horn is then injected high up with the suspension by means of a syringe. Any tendency for the puncture to bleed is stopped by the electric cautery; this is especially useful, when living suspensions of bacteria are employed, for checking any leakage. The abdominal wall, fascia, and skin are sutured in layers. Full asepsis is employed.

The substances used to elicit epithelial phagocytosis are as follows:—

I. *Hydrokollag*.—This is a colloidal suspension of graphite manufactured by de Haën of Hannover. The kind employed was that described as No. 300 by Drinker and Churchill (1927), who appear to be the first to have used this preparation for physiological purposes in England. I am indebted to Dr. Howard Florey for suggesting this preparation as a suitable one; it is also according to his instructions in Sherrington's 'Mammalian Physiology' (1930) that the suspension was prepared. The smallness and regularity of size, and the clarity with which individual particles stand out beneath the microscope, make this an ideal preparation for testing the phagocytic powers of cells. Hydrokollag as received from the makers is sterile on account of the high ammonia content.* Provided it be prepared in sterilised vessels, diluted with sterile saline, and preserved in the cold store, it will keep for many weeks without contamination, as pointed out by Drinker and Churchill (1927).

II. *Carmine*.—Finely ground carmine of good quality (Gurr) is made up to form a 5 per cent. suspension in normal saline. It is best injected with as large a needle as the circumstances permit, and can be sterilised by boiling for 1 or 2 minutes.

III. *Cocoa Butter*.—This may be sufficiently sterilised by placing it in a thermostat at 60° C. for 2 to 3 days; it is injected at body temperature.

IV. *Live Bacteria*.—*Staphylococcus aureus* from an agar slope is suspended in sterile saline. For *Tubercle bacilli* a virulent bovine strain (Cockhorn), grown

* This substance is completely eliminated in the course of preparation.

on Petroff's medium and suspended in 2 per cent. sodium carbonate solution was used.

V. *Dead Bacteria*.—Suspensions of the above were killed by the classic method of heating the suspension at 60° C. for 1 hour.

III. Observations.

Except in the lowest segment the epithelium of the rabbit's vagina is columnar and not stratified. In the cervical region there are two kinds of cells: ciliated and non-ciliated, the former predominating. The non-ciliated cells as shown by Hammond (1925) often give a positive reaction with mucin stains. The epithelium of the middle segment of the vagina is largely non-ciliated and columnar. The cells are relatively big, polygonal (when sectioned horizontally) and contain prominent nuclei at their proximal (*i.e.*, attached) ends. The cytoplasm is often finely vacuolated; with mucic-hæmatestin granular masses giving a positive reaction are sometimes seen within these cells. A film of more deeply staining mucus may be visible on the free surface of the epithelium. The free ends of the cells are often irregular or globoid, and occasionally exhibit blunt projections suggestive of pseudopodia.

It should be pointed out that no conclusive instance of phagocytosis by the ciliated vaginal cells has been noted, my observations referring exclusively to the non-ciliated columnar cells.

Phagocytosis of Hydrokollag.—Injection *per vaginam* of several cubic centimetres of this substance elicits an active phagocytosis of the graphite particles by the vaginal epithelium. Some of the hydrokollag is ingested by leucocytes, but their response is not generally marked. Figs. 1 and 2 (Plate 1) illustrate phagocytosis of this substance by cells of the vaginal epithelium. In the first figure some of the particles lie close to, or against, the free border of the epithelium; in the second the hydrokollag, which is visible in most of the cells, is often situated quite close to the nuclei.

When I first encountered fixed epithelial cells apparently loaded with ingested material I suspected an artefact. It was just conceivable, for instance, that the particles had been moved by the edge of the microtome knife during sectioning. But, if that were so, the "drift" of such particles would be in opposite directions in the case of an O or C-shaped organ; whereas material apparently in the cells of the upper segment might have been transported from the lumen by the knife edge, this would not occur in the case of cells in the lower segment. On examination, however, the pigment was found fairly evenly dispersed throughout the epithelium in any given section. Furthermore, previous

experience (Carleton, 1924) with sections of dust-laden lungs had shown me that particles have to be very large before they are moved by the knife. And when they do move, they leave obvious tracts behind them.

The criteria adopted for clinching the intra-cellular nature of particles are those which I have used elsewhere (1925). They are (i) to try to identify the cell-membrane as outside the particles, and (ii) to note whether some of the particles can be brought by careful focussing into the same focal plane as the nuclear chromatin or nucleoli. Some of the cells in fig. 1 fulfil these requirements; cell boundaries, nuclear outlines and ingested particles are all clearly defined in the same focal plane.

Phagocytosis of Carmine.—This substance is readily taken up by the vaginal epithelium, although the particles are considerably larger than those of the hydrokollag suspension. Fig. 3 (Plate 1) shows a number of large intra-cellular carmine granules, round some of which are distinct cytoplasmic vacuoles. (Photographic reproduction has unfortunately diminished the clarity of these.) These vacuoles afford additional and cogent evidence of the intra-cellular nature of the pigment. Carmine (or hydrokollag) may be taken up by the vaginal epithelium in as short a time as $1\frac{1}{2}$ hours after injection. Injection of one horn of the uterus is often followed by active phagocytosis on the part of the vaginal epithelium, the latter appearing to act as an active interceptor of the injected material during its passage down the vagina.

Phagocytosis of Bacteria.—Suspensions of *Staphylococci* and *B. tuberculosis* were injected after preparation in the manner already described (p. 2). Only a few experiments were made with dead bacteria; no epithelial phagocytosis was observed and the following results are all derived from injections, either into a uterine horn or into the vagina, of living *Staphylococcus aureus*. A few (4) experiments were made with tubercle bacilli; no epithelial response was here noted, possibly because of the very active ingestion of the tubercle bacilli by leucocytes.

Fig. 4 (Plate 2) demonstrates the phagocytosis of *Staphylococci* by the vaginal epithelium; bacteria definitely within columnar cells are shown at X. As with carmine granules, so with *Staphylococci*; cytoplasmic vacuoles may be seen around the ingested material. (These vacuoles need very careful focussing and cannot be seen in the microphotographs.)

The rapidity with which *Staphylococci* disappear after injecting them either into a uterine horn or into the vagina is startling. Six experiments were made in which the bacteria were injected into the vagina, and seven where direct injection of a horn was practised. In the former, undoubted epithelial phago-

cytosis was noted in two experiments, in the latter in only one. The time varied from 6 to 24 hours from the moment of injection to the necropsy. For the reasons already stated, and on evidence produced in the following pages, I regard the epithelial phagocytosis both of pigment particles and of bacteria as undoubted. Why, then, the difficulty of fitting a positive result in *each* experiment? This, I think, is ascribable to the intervention of the white cells. Assessment of the relative activity of epithelial *versus* leucocytic phagocytosis has shown that when the former is marked, emigration of, and phagocytosis by, the leucocytes is scanty. And, inversely, when leucocytes containing intra-cellular bacteria are abundant, the phagocytic ability of the vaginal epithelium seems to be masked and dominated by the voracity of the scavenging cells. This, at any rate, is the provisional explanation offered. It should be possible to confirm (or refute) this suggestion by producing a leucopenia. Experiments intended to bring evidence to bear on this matter are in progress.

Absorption or Phagocytosis of Cocoa Butter and Olive Oil.—Injections of these substances into either the vagina or a uterine horn are sometimes followed by the appearance of fatty globules in both the vaginal and uterine mucosæ. Of the substances examined up to date, fat is the only one which has been found within the non-ciliated columnar cells of the uterus.

Such fat, however, does not seem to be readily taken up by the uterine mucosa. Out of 11 experiments in which one or both horns were injected, positive results were only observed in two, 5½ and 18 hours after injection respectively; furthermore, in both these instances the horn was ligatured close to the *os uteri* before making the injection. This suggests that the process of taking up the fat is a slow one; also, that the injected material is moved out of the uterus by peristalsis before the epithelium has had time to take it up. Is intra-cellular fat absorbed or is it taken up by phagocytosis? The fact that the uterine mucosa has never been noted as phagocytic with any of the substances tested other than fat, suggests absorption. Intra-cellular fat within columnar non-ciliated cells of the uterus is shown in fig. 5 (Plate 2). The globules are strikingly large. Similar pictures were seen in the vaginal epithelium in 5 out of 11 injections. No fat within the uterine and vaginal mucosæ was seen in control preparations.

Possibility of Injected Material Lying in the Inter-cellular Spaces.—It seemed just conceivable that injected particles might be in the cement substance rather than in the cells.* Many histologists (*e.g.*, Kolossow, 1898) claim that definite

* I have examined my material at high magnification and failed to see either bridges or cell-spaces. But it is only fair to say that I did not make use of Kolossow's special fixative.

inter-cellular spaces, traversed by cell-bridges, are found in columnar epithelia as well as in the epidermis, where the existence of such bridges is undoubted.

Dissociation in Ranvier's alcohol was used, the idea being that its solvent action on the cement substance would release the particles from their association with it were they not truly intra-cellular. The cells, however, when dissociated and suitably stained still contain undoubted intra-cellular particles as shown in fig. 6 (Plate 2).

Another and more striking proof of actual ingestion of foreign bodies by the cytoplasm is afforded by horizontal sections of the vaginal epithelium. The polygonal cell-membranes are clearly shown by Heidenhain's iron hæmatoxylin followed by careful differentiation (fig. 8, Plate 2).

The Mode of Phagocytosis.—It has been shown that epithelial cells still attached to their basement membrane and preserving their normal relationships can actively engulf foreign bodies. What is the mechanism? The irregularity of the free ends of the cells, and the blunt projections which they frequently exhibit, suggest pseudopodia. Possibly the cytoplasm is sticky; the fact that it often contains histo-chemically detectable mucin makes this assumption likely. Foreign bodies are therefore presumably held by the cytoplasm on making contact with it. That such bodies are often found close to the nucleus is only explicable on the assumption that the cytoplasm is motile. The curious blunt projections of the cells in fig. 7 (Plate 2) are suggestive of pseudopodia being extruded.

To enter further into the nature of a phagocytic mechanism on a purely morphological basis would only lower this discussion to the level of conjecture. The microscopist labours under a double disability when interpreting physiological processes (such as phagocytosis) on a morphological basis. For he is confronted not only with a fixed and stained section of dead cells and tissues but also with what I may perhaps call a section of time. From the appearances of cells fixed at a certain moment, the observer is tempted to make deductions as to their past and their future. To avoid this mode of interpretation and its attendant pitfalls an attempt is being made to observe vaginal phagocytosis by living cells *in vitro*.

IV. Discussion.

It is usual to regard phagocytosis as the "perquisite" of various motile cells which are generally regarded as mesodermal in origin.

Much discussion has surrounded the derivation of the pulmonary dust- and heart-failure cells which are morphologically indistinguishable. It is impossible to enter into a survey of the literature here. A number of observa-

tions, on a subject in which I am particularly interested, have led me to conclude that both dust-cells and the large elements which appear in chronic pulmonary congestion are formed from the alveolar lining, and are hence epithelial in origin (Carleton, 1918, 1924, 1925, 1925a, 1927). Recent work by Cowdry (1925) on a curious form of broncho-pneumonia in cattle lends support to this opinion. So, likewise, do the remarkable observations of Cappell (1929) on vital staining of the macrophages of the lung.

Cells which, in the organism, are not normally phagocytic are known to become so in tissue cultures. Probably the most curious reversion to cellular-cannibalism *in vitro* is that recorded by Champy (1914). This observer has shown that, in cultures of mammalian retina, the neuroglial elements known as the fibres of Müller voraciously ingest the other retinal elements. The bronchial epithelium is likewise phagocytic *in vitro* (Carleton, 1925). This observation has been strikingly extended by Duthie (1930) who reports that the bronchial mucosa of guinea-pigs can ingest red blood corpuscles and soot.*

Phagocytic powers have been ascribed also to various segments of the male genital ducts. Thus, the cells of the *vas deferens* have been found to be actively phagocytic in respect of spermatozoa after vaso-ligation (Guieyese-Pellissier, 1911). Similarly the epithelium of the *epididymis* (Regaud and Tournade, 1911) and the Sertoli cells of the testicle are supposed to ingest degenerating sex-cells (Regaud, 1901).

My observations relative to the phagocytosis of widely different foreign bodies—varying from carmine and graphite to *Staphylococci*—by the vaginal epithelium would appear to afford the female counterpart to the above.

To summarise, the conclusions forced upon one are that certain mammalian epithelia (mesodermal in origin in the case of the male and female genital ducts) are capable on appropriate stimulation of responding by phagocytosis to the call made upon them. Even in man, there is evidence to show that the alveolar epithelium of the lung plays an important rôle in the defensive response to invasion by dust particles, bacteria and extravasated red blood corpuscles. And, it is quite conceivable that other human epithelia may be possessed of similar potentialities.

Part of this work was done in the Sir William Dunn School of Pathology, Oxford. It is a pleasure to record my appreciation of the facilities afforded me there by Professor Georges Dreyer, F.R.S. The microscopic observations

* On going to press, a paper by Ropes ("Contrib. to Emb.," Carnegie Inst., No. 128, 1930) has come to my notice. It is clearly shown that the ciliated cells of both branchi and trachea in the rabbit are actively phagocytic in respect of carbon particles.

were made in the Physiology Department, Oxford, and I am very grateful for Sir Charles Sherrington's interest in this research.

My thanks are also due to Dr. A. A. Tarkhan, Mr. Jesse Wheal and Mr. Thomas Marsland, who have assisted in the experiments.

V. Summary.

(1) The vaginal epithelium of the rabbit is phagocytic. This capacity appears to be limited to the non-ciliated columnar cells. The ciliated elements—most numerous in the upper vaginal segment—are in this respect inactive.

(2) Carmine, hydrokollag and *Staphylococci* are taken up by the epithelial cells after injections either into the vagina or into a uterine horn. In the latter case, the vagina may behave as an active interceptor of injected material in the course of its passage down from the uterus. No definite instance of phagocytosis by the uterine epithelium has been recorded.

(3) Fats, such as cocoa butter and olive oil may be taken up by both the uterine and vaginal epithelia. Reasons are given, however, for regarding this process as absorption rather than phagocytosis.

(4) The need for stringent criteria in defining phagocytosis has been stressed, and the criteria employed described. They are (i) the ability to bring into the same focal plane some portion of the nucleus and the suspected ingested material; (ii) to identify the cell-membrane as being definitely outside the particles. The presence of cytoplasmic vacuoles around ingested particles such as carmine and *Staphylococci* is additional evidence of phagocytosis.

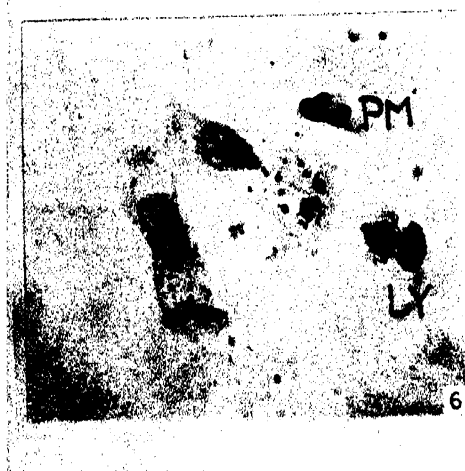
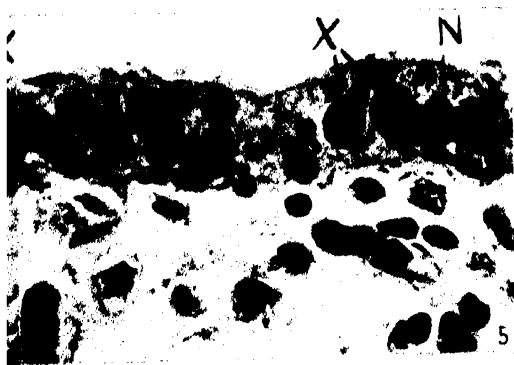
(5) A provisional explanation of the mechanism whereby fixed epithelial cells can actively engulf foreign bodies is given. There is some reason to believe that the free border of the cells—often covered with mucus—is sticky; also that short, blunt pseudopodia may be emitted. The presence of intracellular matter close to the nucleus can only be explained by assuming that the cytoplasm of these epithelial cells is motile.

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(MICROPHOTO : W. Chesterman.)



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EXPLANATION OF PLATES.

All the figures are from untouched microphotographs, for which I have to thank Mr. W. Chesterman. They have been reproduced at the original magnifications. All the photographs, except No. 6, are from sections 8μ thick of the vaginal epithelium of the rabbit.

PLATE 1.

- FIG. 1.—Small crypt showing hydrokollag particles (X) within its cells. From a rabbit which had received two injections (*per vaginam*) of 5 c.c. Killed 7 hours after the last injection. Susa: hæmatoxylin and orange G. ($\times 1000$.)
 FIG. 2.—Another example of the phagocytosis of hydrokollag by the vaginal epithelium. Animal killed 18 hours after a single injection of 7 c.c. *per vaginam*. Most of the columnar cells contain ingested particles. Susa: hæmatoxylin and orange G. ($\times 1100$.)
 FIG. 3.—Phagocytosis of carmine particles (X), many of which can be seen to be adjacent to, and in the same plane as, the nuclei. Further proof of the intra-cellular nature of the carmine is evidenced by the cytoplasmic vacuoles (difficult to discern in the photograph) around the grains of carmine. 1.5 c.c. of a 5 per cent. carmine suspension were injected into the right uterine horn 19 hours before death. The carmine has been actively intercepted by the vaginal epithelium in the course of its passage to the exterior. Susa: hæmatoxylin and orange G. ($\times 1000$.)

PLATE 2.

- FIG. 4.—Phagocytosis by the vaginal mucosa of a suspension of living *Staphylococcus aureus*, injected into one uterine horn 6 hours previously. Bacteria within columnar cells are shown at X. Bouin's fluid: Gram's stain and carm-alum. ($\times 1000$.)
 FIG. 5.—Absorption (or phagocytosis) of cocoa-butter by the vaginal epithelium. Injection of 2 c.c. into one uterine horn has been followed by interception of the fat (in the course of its passage to the exterior) by the vaginal epithelium. Animal killed 18 hours after injection. Intra-cellular fat was not present in the control vagina (3) examined up to date. X = intra-cellular globules of fat; N = nuclei of epithelial cells.
Note.—In the original photograph the oemucated fat and the red-stained nuclei appeared equally black. Hence here the fat has been darkened by retouching the photograph to avoid confusion with the nuclei. ($\times 600$.)
 FIG. 6.—A dissociated preparation of vaginal epithelium from the same experiment as fig. 1. The intra-cellular nature of the carmine in the two isolated columnar cells is clearly shown. Ranvier's alcohol (1 week's treatment); careful dissociation,

followed by staining in dilute hæmatoxylin and orange G. PM = polymorph leucocyte; LY = two lymphocytes. ($\times 1000$.)

FIG. 7.—Edge of one of the vaginal rugæ of the rabbit highly magnified, showing particles within the columnar cells. This, and the succeeding figure, show conclusively that such particles are genuinely intra-cellular, and not wedged in the inter-cellular spaces. Animal killed $1\frac{1}{2}$ hours after an injection, *per vaginam*, of carmine and of hydrokollag. Formol: Heidenhain's iron hæmatoxylin. ($\times 1700$.)

FIG. 8.—Horizontal section of the columnar cells of one of the vaginal rugæ from a similar specimen to fig. 7. Here again the carmine particles are obviously within the cells and not in the cell spaces. Intra-cellular carmine particles are seen at X; the appearance of the particle at XX is due to diffraction. Formol: Heidenhain's iron hæmatoxylin. ($\times 1700$.)

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The Pigmentary Effector System. VI. The Dual Character of Endocrine Co-ordination in Amphibian Colour Change.

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[PLATES 3, 4.]

Part I.—*The Normal Responses of Xenopus laevis.*

In foregoing contributions to this series an attempt has been made to analyse the process of co-ordination in a characteristic mode of behaviour encountered in Amphibia and Reptiles. It is now clearly established that the pituitary gland is an essential agency in determining Amphibian colour change. Accepting this conclusion, it is still possible to entertain the hypothesis that the secretion of the pituitary gland is merely a condition of the response of the melanophores to nervous impulses in efferent fibres supplying the skin. The present investigation is an attempt to subject this possibility to more rigorous examination. The direct innervation of Amphibian melanophores has not been established by histological examination, and the time relations of pigmentary effector activity in Amphibia do not suggest the intervention of a peripheral nervous mechanism. As shown in the last communication of this series, the melanophores of Reptiles are directly innervated. Experiments on

stimulation and section of peripheral nerve trunks have led investigators on Amphibian colour change to ambiguous results, which have been extensively criticised elsewhere. The chief source of misunderstanding arises from the fact that peripheral nerves in general contain vasomotor components. In work on the chameleon it has been possible to demonstrate segmental effects of nervous origin, by employing as a stimulus to generalised pallor an agency for which the receptor surface is localised. For further analysis of co-ordination in Amphibian colour change it now becomes necessary to undertake a more precise definition of the receptive field. A visual response naturally suggests itself as the most convenient type for this purpose. In the European frog (*Rana temporaria*) various factors, including humidity and temperature, significantly co-operate with illumination to determine pigmentary effector activity. The South African clawed toad is an aquatic Anuran whose chromatic responses are almost entirely determined by photic stimuli. This circumstance has made it possible to explore the problem of co-ordination more thoroughly than in previous researches recorded in this series of communications.

In *Xenopus* as in other Anura there are three types of pigmentary effector organs. These are (a) the epidermal melanophores, (b) the dermal melanophores, and (c) the xantholeucophores. The dermal melanophores are the most important agents in the visible reaction. Their condition can easily be followed by microscopic examination of the web. In what ensues attention is directed to the dermal melanophores, unless otherwise stated.

Those who have studied pigmentary effector activity in the English common frog are agreed that humidity and temperature are eminently significant external agencies. In consequence the influence of light assumes a subordinate rôle. For the platana light reflected from the field of vision is the all-important factor. To avoid periphrasis the word "background" will be used in this communication to denote the field of vision. Strictly speaking it is inaccurate, because the eyes of *Xenopus* are situated on the dorsal aspect of its head. In the experiments which follow the behaviour of normal animals will be contrasted with that of eyeless animals. From the latter the entire eyeballs were removed. To define with greater precision the condition of the melanophores we have employed a method adopted by Hower (1923) at the suggestion of the senior author. Arbitrary numerical symbols have been applied to five stages of melanophore contraction and expansion, selected as suitable for convenient identification and defining the state most characteristic of certain equilibrium conditions. These are indicated in fig. 1. Photomicrographs are reproduced in Plate 4. Unfortunately it is difficult to photo-

graph an expanded melanophore as it appears in the living condition, because only a small part of it can be focussed at the same time.

Generally speaking, there is considerable uniformity of the pigment cells

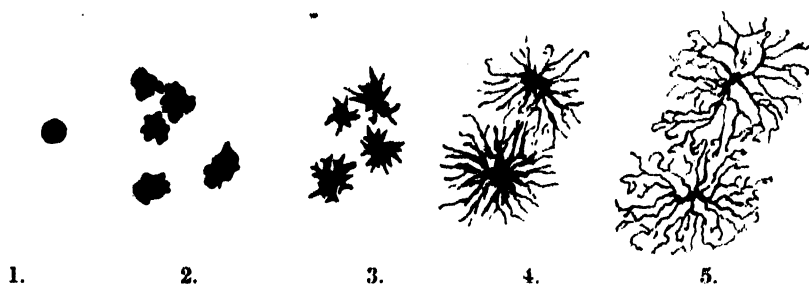


FIG. 1.—Melanophore Index.

in adjacent areas of the web at any given moment. When some are seen in one condition and others in another, a mean value is given. For instance, a very dark animal may have islands of stage 4 on a general background of stage 5. The melanophore index of such an animal is denoted 4.5. This method of dealing with colour response has made it possible to demonstrate differences which are not readily detectable from the macroscopic appearance of the animals. Although the symbols selected are entirely arbitrary, the readings of different observers do not significantly differ. Since the observations recorded in the experiments described in this section were actually carried out, the same series of experiments has been conducted by students as class work for three consecutive years. The results of students' observations have always been confirmatory of the conclusions which will be set forth.

The containers used for the experiments were square tin receptacles 32 cm. broad and 7 cm. deep. They were either varnished to present a dull metallic surface ("neutral background") or painted on the inside with a hard black or white enamel. Usually five animals were placed in one container. The water was changed at regular intervals, when the animals were fed with raw meat once a week. Experiment showed that the animal's field of vision was almost entirely occupied by the sides of the vessel. Only the rim of the floor and no part of the cover were included in the visual field. The cover was a plate of thin, flat glass, 35 cm. square. Three sorts of covers were used to allow for bright, medium or dim incident illumination. These were respectively unpainted, painted with white enamel, and painted with black enamel. The black and white plates were not completely light-proof. To control their possible relation to the visual field two precautions were taken. White and

black tops were fixed above the plain glass tops at a sufficient distance to admit diffuse light, and a white plate was always placed beneath a dark plate, when the latter was used to secure dim illumination.

Clawed toads kept in white containers in diffuse light become pale within 24 hours, though they do not attain maximum pallor until several days have elapsed. Kept in black containers they become dark in a few hours. It has long been known that the characteristic white-"background" response of fishes and amphibia is abolished, if the eyes are removed, or the optic nerves severed. Previous workers have described the eyeless frog as a "dark" animal. After removal of the eyes *Xenopus* remains dark on a white background; but the eyeless animal is never quite so dark as a normal animal kept on a black background. When normal and eyeless animals are kept in a large black tank, there is no difficulty in distinguishing the majority of the eyeless animals by their macroscopic appearance. Comparing the animals kept under different conditions of illumination, a somewhat paradoxical impression is gained from the macroscopic appearances. The most extreme pallor was found in animals placed on a white background in dim light, whereas the most extreme darkening of the skin was manifest in animals placed on a black background in bright light. This seeming paradox appeared to be consonant with observations on *Urodele* larvæ, which have been placed on record by Laurens (1915-16). Laurens found that in bright light eyeless individuals become darker, and seeing individuals at first become darker and then paler. Laurens distinguishes between a *primary* or non-visual response to light by expansion of the melanophores, and a *secondary* response, superimposed upon the former, dependent upon the eyes, and influenced by light in the opposite direction.

Statistical treatment of the microscopic appearance of the web confirmed the impressions gained from the visible appearance of the animals. In the data cited in the ensuing protocols the mean value of the melanophore indices of a group of individuals is given. The number of individuals and the standard deviation of the mean difference is stated in each case. Experiments I, II and III show that removal of the eyes abolishes both the white background and the black background response. The condition of the dermal melanophores in the eyeless animal and in animals kept in darkness, as shown in experiment V, is intermediate between the extreme conditions usually described as "contracted" and "fully expanded." The data of experiments I to V also indicate that in *Xenopus* there is a slight degree of primary reactivity of the type seen in *Urodele* larvæ. This is analogous to the reptilian type of photic

reaction, but the possible range of this primary reactivity is very small compared with the secondary or co-ordinated activity, which is dependent upon the eye. The threshold of illumination requisite for the manifestation of the primary response is much higher than for the secondary. Thus, in dim light, the secondary response on white background is not diminished by the primary reactivity, whereas in bright light the black background response is reinforced by the primary reactivity.

Experiment I.

Exposed for 24 to 28 hours in diffuse light, 10 days after operation in case of eyeless toads. Temperature 19° to 20° C.

	White background with glass tops.	Black background with glass tops.
Eyed	1.90 ± 0.04	4.54 ± 0.07
Number of individuals	48	48
Eyeless	3.13 ± 0.13	3.13 ± 0.15
Number of individuals	48	48

Experiment II.

Exposed for 24 hours to diffuse light at 19° to 20° C. 14 days after removal of eyes in case of eyeless animals. Eight individuals in each batch.

	White covers.		Black covers.	
	White background.	Black background.	White background.	Black background.
Eyed	2.0 ± 0.00	4.6 ± 0.13	1.65 ± 0.16	4.25 ± 0.15
Eyeless	3.13 ± 0.30	3.0 ± 0.31	2.75 ± 0.18	2.75 ± 0.23

With the dimmer illumination (black covers) the consistent shift towards greater pallor is seen in another experiment, in which the background was a varnished metallic surface which reflected light to a considerable extent. This is set forth in the table in connection with experiment III.

Experiment III.

Exposed 24 to 48 hours diffuse light at 19° to 21° C., eyeless 14 days after operation. Eight toads in each batch. Neutral, that is, metallic background.

	Glass cover.	White cover.	Black cover.
Eyed	2.6 ± 0.24	1.5 ± 0.25	2.4 ± 0.35
Eyeless	3.4 ± 0.17	2.4 ± 0.17	2.4 ± 0.17

Before proceeding to give further proof of this restricted degree of primary reactivity, which consistently emerged in our earlier experiments, we may pause to comment upon one issue. The intermediate condition of the eyeless animals in the early stages after operation might be attributed to stimulation resulting from necrotic changes in the degenerating optic fibres. We have, therefore, tested the eyeless animals repeatedly. An experiment carried out on an extensive scale, after the lapse of sufficient time to ensure complete degeneration of the optic fibres, is here included to demonstrate the fact that conclusions drawn from experiments on animals 14 days after operation are not invalidated by the possibility stated.

Experiment IV.

Exposed 24 hours in diffuse light at 16° C., 6 weeks after removal of eyes of eyeless toads.

	Black background, black tops.	White background, white tops.
Eyed	4.82 ± 0.07	1.8 ± 0.08
Number of individuals	28	27
Eyeless	2.8 ± 0.13	3.5 ± 0.13
Number of individuals	28	27

In darkness the normal animal approximates to the condition of the eyeless animal as seen in the ensuing experiment.

Experiment V.

At 17° C., 6 weeks after operation in case of eyeless toads.

	Complete darkness.	Bright illumination.
	60 hours.	10 hours.
Eyed, on white background, 12 toads	2.7 $\pm$ 0.24	2.1 $\pm$ 0.08
Eyed, on black background, 12 toads	3.0 $\pm$ 0.18	5.0 $\pm$ 0.18
Eyeless, 48 toads	3.0 $\pm$ 0.11	3.8 $\pm$ 0.09

The data tabulated under experiment V provide conclusive evidence for the view that the neutral condition of the melanophores is intermediate, and that, superimposed on the major, visual photic response, there is in *Xenopus* a slight degree of primary reactivity of the chameleon type. It is seen in I to IV that the normal toad subject to dim illumination on a white background becomes darker, and on a black background paler than in bright light. It is also seen that the eyeless animal is significantly darker in bright light. Using the same standard of arbitrary numerical symbols to denote different degrees of melanophore expansion, we may say that the range of the primary reactivity is, at a maximum, only 10 per cent. of the range of secondary reactivity.

Humidity is not a significant factor in colour change in *Xenopus*, as shown in the following experiment. The point is hardly worthy of detailed investigation, as the animal will not live very long out of water, and if placed in a dry container it is kept moist by its own skin secretion for many hours.

Experiment VI.

Eyeless toads for 24 hours on neutral background in diffuse light at 17° C.

	Mean value.	Number of toads.
Wet	3.4 $\pm$ 0.18	16
Dry	3.1 $\pm$ 0.15	16

Between 12° C. and the lethal upper limit (about 33° C.) we could detect no significant temperature effect on colour change in *Xenopus*. Below 10° C. there is a definite tendency to darkening of the skin. Thus the general type of effect is similar to that seen in most vertebrates which display chromatic response. The noteworthy feature of *Xenopus* is the wide range of temperature over which the chromatic function remains little affected.

From the data presented above it appears that the dermal melanophores of normal individuals of *Xenopus laevis* are contracted when the animals have been kept for at least 24 hours on a white background between 15° C. and 30° C. They are expanded after exposure to a black background for the same period, and in complete darkness they assume an intermediate degree of expansion, preserved in diffuse light by the eyeless animal independently of the background. This visual response is superimposed upon a very restricted degree of primary reactivity of the reptilian type, not dependent on the eye.

The conclusions which have been set forth above were confirmed by experiments carried out after a lapse of 12 months. The only new point which emerges is that the equilibrium value for eyeless animals a year after operation is rather lower, i.e., as far as the microscopic appearance of the dermal melanophores is concerned they tend to approximate more to the condition of the pale animal. Macroscopically they show no perceptible difference. The following experiment was undertaken after an analysis of the time factor in colour change had emphasised the necessity of prolonged exposure in establishing the true condition of equilibrium. Illumination was continuous and the record was taken after 3 weeks' exposure. The mean temperature was 16° C.

	White background.		Black background.	
	Bright.	Dim.	Bright.	Dim.
Normal	1.25 ± 0.09	1.13 ± 0.07	4.54 ± 0.09	4.58 ± 0.12
Number of toads	24	23	24	24
Eyeless	2.8 ± 0.15	2.15 ± 0.17	2.9 ± 0.09	1.9 ± 0.16
Number of toads	20	20	20	20

In these data the *primary* response of the eyeless toads is brought into sharp relief. Indeed the effect was macroscopically recognisable.

Observations carried out on *Rana fuscigula* confirm the expectation that the essential characteristics of the photic response in *Xenopus* are shared by other Anura. In the following experiment it is seen that there is a visual response in *Rana fuscigula*. As in *Xenopus* the eyeless animal, though relatively dark in its macroscopic aspect, is not as dark as the normal animal in optimum conditions for melanophore expansion. When the condition of the melanophores is recorded in the manner adopted in observations on *Xenopus*, the eyeless animal is seen to be intermediate. In this experiment the temperature

was 16° C. and the records were taken 6 weeks after operative removal of the eyes.

	White container, bright illumination.	Black container. dim illumination.
Eyed	1.0 ± 0.21	4.4 ± 0.22
Number of individuals	5	5
Eyeless	3.4 ± 0.40	3.6 ± 0.22
Number of individuals	5	5

Workers on the European frog have emphasised the importance of humidity and temperature as external agencies contributing to pigmentary effector activity. The significance of humidity is seen in the following protocol. The temperature in this case varied between 19° C. and 20° C., and the record was taken after 3 days' exposure.

	Wet.	Dry.
White container, bright illumination	2.4 ± 0.39	2.1 ± 0.27
Number of individuals	8	10
Black container, dim illumination	4.7 ± 0.11	4.4 ± 0.16
Number of individuals	10	9

Humidity acts on pigmentary changes in the Cape frog in the same sense as it influences colour change in the English common frog, though it is perhaps a less powerful factor. The influence of light on the colour changes of the frog has been less emphasised by European than by American workers. Intelligible reasons for this are now evident. One is that in different species of Anura the relative importance of light, humidity and temperature varies considerably. Another is the fact that the primary and secondary responses are opposed to one another. To bring into sharp relief the purely visual response it is first necessary to control the effects of humidity and temperature. It is also essential to adjust the intensity of illumination, so that the primary response of the melanophores to light does not antagonise the co-ordinated response for which the eye is the receptor organ. When these conditions are fulfilled the result can be predicted with a high measure of confidence.

The most vital issue which emerges from this preliminary enquiry is the circumstance that the eyeless animal displays neither maximum pallor nor maximum darkening of the skin. Previous observers on Amphibian pigmentary activity have noted that the eyeless animal remains dark on a white

background. This is true in so far as the eyeless animal is much darker than a normal animal kept on a white background. It is also true that it is paler than a normal animal kept on a black background. This cannot be explained on the simple hypothesis that light reflexly inhibits the activity of the pituitary gland. If that were so, the eyeless animal should be darker than the normal animal kept on a black background. The experiments to be recorded in the ensuing sections were undertaken to elucidate this anomaly.

Summary of Part I.

(1) Within a wide range of temperature (12° to 32° C.) normal individuals of *Xenopus laevis* become dark if placed in light in a situation such that the field of vision is occupied by a surface which absorbs light ("black background") and become pale if placed in light in a situation such that the field of vision is occupied by a surface which scatters and reflects light ("white background").

(2) Eyeless individuals do not display this type of reactivity, but, like normal animals subjected to prolonged exposure to total darkness, remain in an *intermediate* condition due to the partial expansion of the dermal melanophores.

(3) In eyeless animals the melanophores are more expanded in light than in darkness. There is thus a "primary" reactivity of the pigmentary effector system independent of the eyes and tending in the opposite direction to the "secondary" co-ordinated response for which the eye is the receptor organ. The primary response is much less considerable in extent than the visual response.

(4) The threshold of illumination for the "primary" reaction to light is higher than that for the secondary. The optimum condition for pallor in normal animals is therefore provided by dim light reflected from a light-scattering surface occupying the field of vision. The optimum condition for darkening is a light-absorbing visual field with bright illumination from above.

Part II.—The Time Relations of Colour Change in Xenopus laevis.

For the present we shall disregard the primary reactivity which may depend on the direct response of the pigmentary effector organ to the stimulus of light, or may be a co-ordinated response for which the receptor is some photo-sensitive end-organ in the skin. The secondary or visual reactivity, on the other hand, must involve a co-ordinating system. This may be endocrine, nervous or both. The fact that eyeless animals are neither completely pale nor completely dark suggests that the white background and black background responses involve separate agencies for the co-ordination of stimulus with

response. The twofold character of the visual response may be explained by either of the following hypotheses or by a combination of both :—

- (a) That there are two kinds of photoreceptive elements in the retina with different thresholds of excitation, one tending to produce inhibition and the other tending to produce activation of the same mechanism affecting the melanophores.
- (b) That there are two separate mechanisms affecting the melanophores in the opposite sense, one predominating over the other according to the number of photoreceptors stimulated, and one responsible for the contraction, the other for the relaxation of the melanophores. In discussing this hypothesis the agency which is responsible for the white background response will be referred to as W, and the agency which is responsible for the black background response will be referred to as B.

A study of the time relations of the response supports the second hypothesis. It also provides information concerning the nature of the co-ordinating agency or agencies involved. In any mode of behaviour the interval which elapses between the application of the stimulus and the execution of the response is generally the sum of at least three components. These are the latent period of the receptor organ, the time occupied by the process which occurs in the process of co-ordination and the time occupied in bringing the effector organ itself into play. The reaction time of Amphibian melanophores may be judged from observations on the action of drugs and autoacids. It is certainly less than an hour, and the latent period of the retina and propagation within the C.N.S. are negligible in comparison with this. On the other hand, it will be seen that the secondary responses take many hours to reach completion.

We shall now consider separately the time relations involved in :—

- (i) passing from the black- and white-background response to the "intermediate" condition in total darkness ;
- (ii) transition to the white- and black-background response from the intermediate condition consequent on prolonged exposure to darkness ;
- (iii) complete reversal of the white- and black-background response in both directions.

It must be borne in mind that the numerical symbols applied to different configurations of the dermal melanophores are quite arbitrary. Though some

insight may be obtained from a consideration of the intervals which elapse between equilibrium conditions and the intercalation of subnormal or super-normal phases, no significance can legitimately be attached to the gradients of the curves.

a.—*Transition to the Intermediate Condition in Darkness.*

The first problem to be investigated was the time taken by dark and pale animals to reach the intermediate condition in complete darkness. Previous experience had shown that this was considerable, and that pale toads required a more protracted exposure to darkness than dark ones before equilibrium is established, *i.e.*, before the *eyeless* condition is attained. In the first experiment (fig. 2) 11 pale toads in white pots and 11 dark toads in black pots were

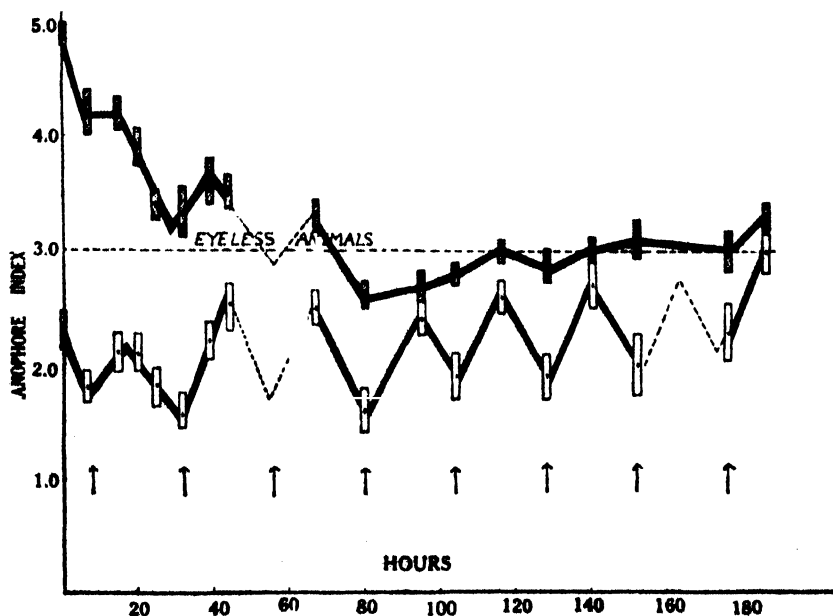


FIG. 2.—Dark room experiment at 22° C. Upper series toads in black containers—originally dark animals. Lower series toads on white containers—originally pale animals. Arrows represent 24-hour intervals. Midnight to midnight.

placed in a large light-proof and well-ventilated box in the dark-room. The use of the box disposed of the necessity of exposing any individual to light for more than a few seconds when observations were taken. In this experiment there are three significant features which at once emerge from the graphical record. First, that the dark animals attain the eyeless equilibrium much more readily than the pale ones. Second, that there is a decided tendency for

the melanophores to be more contracted at midnight, suggesting a diurnal variation analogous to that recorded for the primary photic reaction of Crustacea by Keeble and Gamble (1902 and 1905). Third, that dark animals, after reaching the intermediate condition within 40 to 50 hours, become paler than the eyeless animal, and remain so until about 120 hours after beginning of experiment. That is to say, they do not settle down to the eyeless equilibrium condition till about the same time as the toads which were initially pale. To obtain further confirmation on the last two points, *i.e.*, the diurnal variation and the subnormal phase of the initially dark toads, the experiment was repeated at a different temperature. In the second experiment 12 toads in each series were employed, initially pale animals being placed in black pots and initially dark animals in white pots immediately before placing in the dark-room. In this second experiment the time required to attain equilibrium on the part of the initially pale toads was even more protracted than in the first. A part only of the experiment is reproduced, but the diurnal variation was consistent throughout the entire series of observations (fig. 3). While the

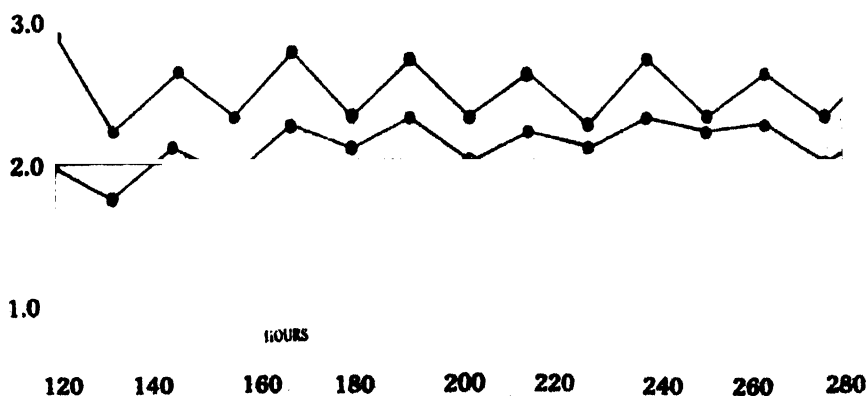


FIG. 3.—Part of an experiment showing transition from pale (lower) and dark (upper) condition to the intermediate state in darkness. This shows regular diurnal variation, the arrows indicating midnight.

variation is not significantly outside the range of probable error for two successive points on the curve, the complete consistency of the two curves, and the perfect regularity of the result, leaves no doubt concerning the existence of the diurnal change. It is not without interest to note that the rhythm of the dark-room toads is in the same sense as the normal diurnal rhythm result-

ing from the primary response. This at first suggests that the mechanism involved is a "conditioned" one in Pavlov's terminology. In the second experiment the subnormal phase of the initially dark toads was quite pronounced, and complete equilibrium of the initially pale animals took about a fortnight.

b.—Transition from Darkness to White and Black Backgrounds.

Two experiments were carried out on the same two series of animals as were employed in those represented in figs. 2 and 3. Fig. 4 is a continuation of fig. 2,

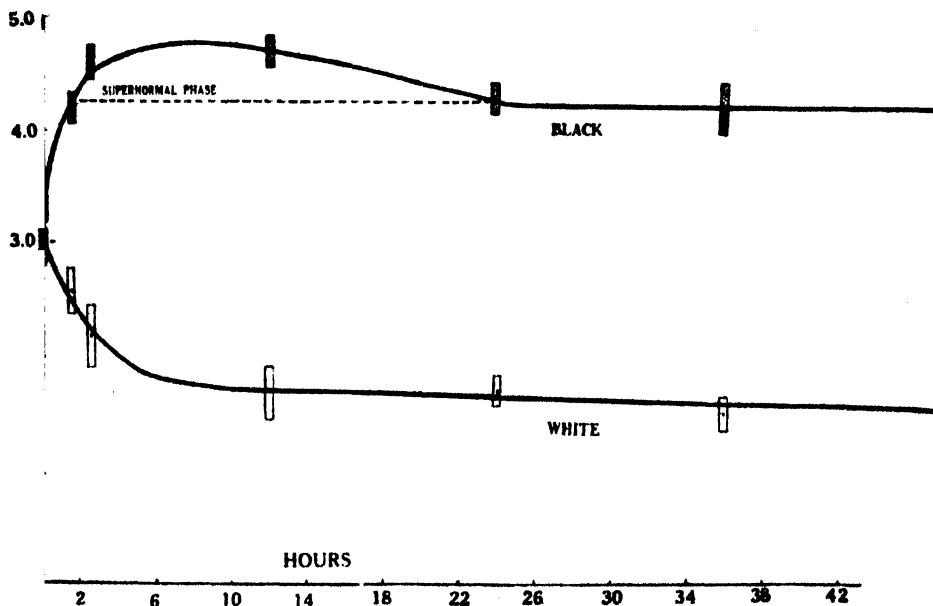


FIG. 4.—Transition from intermediate condition in darkness to dark and pale condition in light. Upper series in blackened containers, lower series in white containers. Temperature 22° C.

the origin being taken as the point of illumination to continuous diffuse artificial light. On a black background the melanophores attain in about an hour a condition of average expansion similar to that of their final equilibrium condition. They continue to expand further for a while. Then they assume their final equilibrium about the time when the series placed on white background attained maximum pallor asymptotically 25 to 30 hours after the beginning of the experiment. In a repetition of this experiment on a second series of individuals to ascertain whether the apparent existence of a supernormal phase in the series placed on black backgrounds was the fortuitous

result of reinforcement by the diurnal variation, the existence of the supernormal phase was very decisively confirmed. The following protocol makes this evident :—

Time.		White background.	Black background.
h.	m.		
0	00	2.7 $\pm$ 0.22	2.5 $\pm$ 0.19
1	50	2.75 $\pm$ 0.18	3.5 $\pm$ 0.15
3	00	2.5 $\pm$ 0.19	4.1 $\pm$ 0.15
4	00	2.4 $\pm$ 0.14	4.2 $\pm$ 0.16
5	30	2.3 $\pm$ 0.13	4.4 $\pm$ 0.16
6	55	2.18 $\pm$ 0.16	4.4 $\pm$ 0.15
7	55	2.18 $\pm$ 0.10	4.6 $\pm$ 0.14
21	25	2.9 $\pm$ 0.00	4.5 $\pm$ 0.15
24	55	2.0 $\pm$ 0.00	4.4 $\pm$ 0.19
Equilibrium value 150 hours..		1.9 $\pm$ 0.08	$\pm$ 0.12

It is worth while pausing at this point to compare the time relations of the transition from darkness to white and black background respectively, and the reverse operation as recorded in the preceding section. From these experiments it is seen that the time taken to reach equilibrium in passing from the black-background equilibrium to the intermediate state is significantly greater than in the reverse process, viz., in passing from the intermediate condition characteristic of equilibrium in darkness to the black-background response in light. The time taken to pass from the white-background equilibrium to the intermediate condition in darkness is significantly greater than the time taken to pass from the intermediate condition in darkness to white-background equilibrium in light. The time required to pass from black-background equilibrium to the intermediate condition is much less than the time required to pass from the white-background equilibrium to the intermediate condition in darkness. The time required to pass from the intermediate condition in darkness to black-background equilibrium in light is less than the time required to pass from intermediate condition in darkness to white-background equilibrium in light. These conclusions may be epitomised by the aid of the following symbolism where *b*, *w* and *i* represent respectively the equilibrium conditions for black background, white background, and darkness, the arrows the direction of the process, and the usual conventions for inequalities are employed.

$$b \rightarrow i > i \rightarrow b$$

$$w \rightarrow i > i \rightarrow w$$

$$b \rightarrow i < w \rightarrow i$$

$$i \rightarrow b < i \rightarrow w$$

These results are totally inexplicable in terms of hypothesis (a) set forth in the introduction, and the evidence of two separate mechanisms of discharge is reinforced by the existence of the subnormal and supernormal phases. If mechanisms B and W are both brought into play when the eye is illuminated, and the final result depends on the number of photo-receptors stimulated, the slower rate at which W subsides would necessitate a subnormal phase in passing from black-background in light to darkness, while the slower rate of development of W would necessitate a supernormal phase in passing from the intermediate condition in darkness to the black-background equilibrium.

c.—*Reversal of Background Response.*

We have now to consider what happens when animals which have attained the white-background equilibrium are transferred to a black-background and *vice versa*. Here, as before (see fig. 4), mechanism W comes into play much more slowly than mechanism B; in other words, equilibrium is reached more rapidly in passing from white to black than from black to white. As would be expected, it takes much longer to attain equilibrium in passing from these extremes than in passing from the intermediate state to either condition. The time taken to pass from the intermediate level to the equilibrium state when the backgrounds are reversed in light is significantly greater than the time taken to pass from the intermediate condition of animals that have been kept in darkness to either type of background equilibrium. Thus if i_1 and i_2 respectively denote the intermediate condition of the melanophores of an animal kept in darkness and an animal which is in process of passing from black- to white-background in light or *vice versa*, then

$$i_2 \rightarrow w > i_1 \rightarrow w$$

and

$$i_2 \rightarrow b > i_1 \rightarrow b.$$

On the other hand, it takes less time to pass from white-background equilibrium to the black-background equilibrium in light than it does to pass from the white-background to the intermediate equilibrium in darkness ($w \rightarrow b < w \rightarrow i_1$). On the hypothesis of two separate efferent mechanisms B and W, this result is again explicable. The first reaction involves the subsidence of W only, whereas the second result involves the subsidence of W and the subsidence of B.

Two experiments were performed and the results were perfectly consonant. Fig. 5 is based on the same series of animals as those used in the experiment on which fig. 2 is based. It will be noticed that there is no supernormal

phase in passing from white-background equilibrium to black-background equilibrium.

The entire series of data here presented are incompatible with the hypothesis

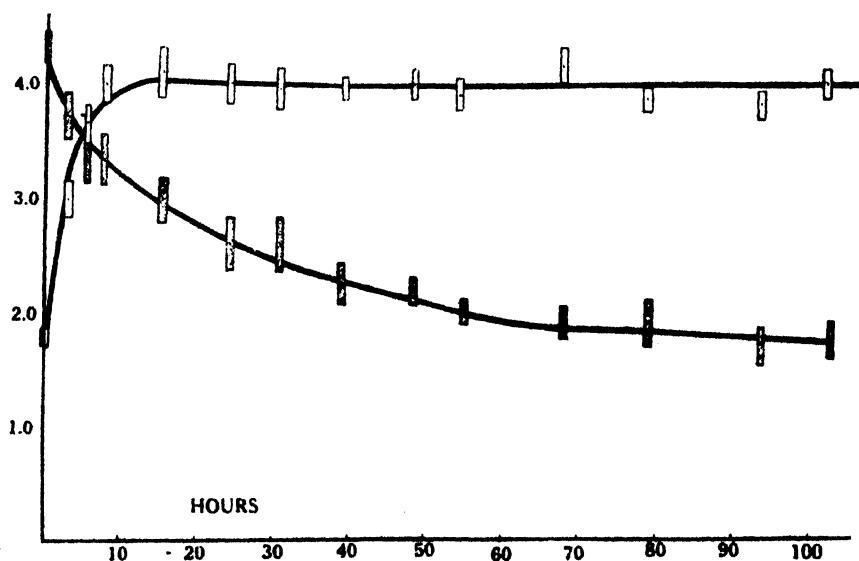


FIG. 5.—Reversal from white to black and black to white "background." Temperature 17° C.

that a single efferent mechanism, viz., the fluctuating secretion of the melanophore stimulant of the pituitary gland, underlies the background responses of *Xenopus*. There may or may not be two sets of receptors whose activation tend to produce opposing results. For the present it is an economy to assume that the number rather than quality of receptors brought into play is the significant factor. [If this view is correct, it should be possible to establish a level of intensity at which a normal animal would become dark on a white-background. Till this has been done, we cannot legitimately dismiss the possibility pointed out to us by Dr. Dale, who suggests that the black-background response is a contrast effect.] In any case, there must be separate efferent mechanisms—using the term efferent without restriction as to endocrine or nervous character of the process—underlying the white- and black-background responses. It is therefore possible to outline a working hypothesis in some such terms as these. When an animal kept in total darkness for some time is placed on a black-background the small amount of light reflected from its surroundings stimulates some photo-receptors, bringing into play mechanism B, i.e., increased output of pituitary secretion, and mechanism W at present

unknown. At first the effect of B overrides W, but as more photo-receptors are brought into play, i.e., when the field of vision is occupied by a light-scattering surface, the output of B reaches a limit before W, so that W overrides B. [Otherwise, adopting Dr. Dale's suggestion that the black-background response depends on the fact that the only retinal elements stimulated are those in the floor of the eyeball, it is possible that the latter bring B alone into play.]

Alternatively, the small amount of light reflected from a light-absorbing surface may inhibit W more than B. Then as more receptors are brought into play the inhibition of B becomes more complete than the inhibition of W.

Summary of Part II.

(1) An analysis of the time relations of the secondary response involved in the transition (i) from the black- to white-background response and *vice versa*, (ii) from the intermediate condition in darkness to the white- or black-background response and *vice versa*, provides evidence that the dermal melanophores of *Xenopus laevis* are subject to a double endocrine control.

(2) There is a diurnal rhythm of colour change in *Xenopus* independent of the visual receptors.

Part III.—The Relation of the Pituitary Gland to Colour Change in Xenopus laevis.

In seeking to elucidate two separate endocrine factors in the secondary photic response the first issue which presented itself for enquiry was the identification of the agency controlling the black-background response. The secretion of the posterior lobe of the pituitary gland has already been proved to be concerned in the darkening of the skin in Amphibia. Accordingly at an early stage a large stock of hypophysectomised animals was prepared for future use. A few preliminary remarks must therefore be made with reference to operative procedure. At the same time a brief reference may be made to the technique employed for estimating the potency of extracts of the posterior lobe of the pituitary gland.

(a) *Operative Removal of the Pituitary Gland.*—The method employed was in principle essentially similar to that described by Hogben for the removal of the pituitary gland in other Anura and in Urodeles. In *Xenopus* it is not possible to see the outline of the pituitary gland through the thin translucent base of the skull as in other Anura and in Urodeles. To locate its position

without extensive exposure it is necessary to apply the dental drill to a spot in the middle of the anterior margin of the common Eustachian orifice. Before doing so a median incision is made in the mucous membrane which is scraped away from base of skull. This procedure usually leads to loss of a good deal of blood if other precautions are not taken. Bleeding can be prevented by cauterising two large superficial vessels with a small electric cautery. Owing to the adherence of the mucous membrane it is easy to tear away large pieces of tissue in applying the dental drill. The mucous membrane was accordingly held back by a pad of rubber which covered the floor of the mouth. Little mortality occurred in operations during the cooler months. A high death rate ensued after removal of the pituitary, if the operation was performed during the hot season from November to March. In either case animals surviving the first 3 weeks after operation live indefinitely in the laboratory, and have been kept under observation for more than 2 years. After 3 weeks they were fed regularly once a week. The water was changed after feeding. Most of the operated animals employed in this investigation had been kept in the laboratory after operation for a much longer period of time than those which have been tested in previous investigations of the same kind.

In preparing stocks of operated animals it was decided at the outset to prepare individuals from which the whole gland and others from which the anterior lobe only had been removed in equal numbers. The latter were originally intended to serve as controls. It will be seen later that they provided ulterior evidence bearing on the rôle of endocrine agencies in controlling pigmentary changes in *Xenopus laevis*.

(b) *Injection of Pituitary Extracts*.—In experiments involving injection of pituitary extracts a new departure has been made. Hogben and Winton have recorded the effects of injecting posterior lobe extracts into normal and hypophysectomised frogs. Their comparison of the reaction of the two groups was based on the minimal dose requisite to produce a macroscopic effect. In experiments recorded in this section a comparison of the effects of injecting pituitary extract into normal and hypophysectomised platanas was based on a more refined procedure developed in the course of this investigation and already described in detail by Hogben and Gordon (1930). This method involves plotting a curve for the variation of the melanophore index with time after injection. It is a more delicate instrument for two reasons. First, as has been shown in the preceding sections, the microscopic appearance of the melanophores can be described with greater precision than the macroscopic effect. Secondly, both the intensity and persistence of the effect are deter-

mined by the amount of active substance injected. The first of these merits further comment. Although it is true to say that in equilibrium conditions the background response of the dermal and epidermal melanophores is the same in *Xenopus laevis*, the rate of change is not the same for both; and though both react to pituitary extracts in the same sense their respective thresholds are not identical. For these reasons it is possible to encounter considerable discrepancies between the macroscopic appearance and the microscopic state of the dermal melanophores in experiments involving injection of pituitary extracts. There is no doubt that these discrepancies can be explained in the main by two circumstances. One is that the epidermal melanophores do contribute significantly to the macroscopic effect. The other is that epidermal melanophores are relatively much more numerous in some individuals than others. In some animals they are so few in number that they are difficult to find. In others they are more numerous than the dermal melanophores. For this reason attention has been directed almost exclusively to the dermal melanophores. To what extent the xantholeucophores play a significant part in the macroscopic effect it is difficult to say. No such consistency in their behaviour, such as that described in *Rana*, was detected in *Xenopus laevis*. In very pale animals that have been kept for a long time on a white background, and generally speaking in hypophysectomised animals, they are seen to be completely expanded. They are also sometimes seen to be very completely expanded in animals whose macroscopic appearance is as dark as possible. These discrepancies may be due to the fact that their rate of reaction is different from that of the melanophores or to the influence of agencies which do not exercise a noticeable effect upon the latter. In some animals they are so inconspicuous that it is not easy to observe how they behave.

(c) *Effects of Operative Removal of the Gland.*—After complete removal of the pituitary gland, *Xenopus laevis* remains pale on a black background indefinitely. Hypophysectomised animals and control series have been kept under observation for a period of more than 2 years. In the course of parallel investigations carried out in this laboratory (Charles, 1931), in connection with metabolic changes accompanying pituitary removal, and pigmentary effector activity, stocks of from two to three hundred animals have been kept under observation. Though the macroscopic appearance of hypophysectomised toads when kept on a black-background would be described as *pale*, a microscopic study of the melanophores indicates that normal animals kept on a white-background are paler. There is a slight but significant difference in the melanophore index of the two groups. The melanophore index of normal toads after prolonged

exposure to white-background is, moreover, significantly lower than that of the completely hypophysectomised animals, also placed on a white-background. This difference is shown in the accompanying table which also discloses the fact that the primary response is independent of the pituitary gland.

Reactions of Normal and Hypophysectomised Toads.

16° C. continuous illumination 10 days.

	White-background.	Black-background.	Darkness.
Normal toads	1.25 $\pm$ 0.09	4.54 $\pm$ 0.09	—
Number	24	24	—
Hypophysectomised (both lobes) 9 months after operation	1.64 $\pm$ 0.13	1.40 $\pm$ 0.13	1.30 $\pm$ 0.13
Number	14	15	15
Hypophysectomised (anterior lobe only), 4 months after operation	4.4 $\pm$ 0.13		
Number	13		

This slight difference in the behaviour of normal animals, exposed to a white-background and hypophysectomised animals exposed to the same conditions, would be inexplicable on the assumption that the co-ordination of colour response in *Xenopus* is solely determined by the activity of the posterior lobe of the pituitary gland. On the other hand, the experiments already recorded have conclusively shown that there are two endocrine agencies involved in the pigmentary responses of *Xenopus*. It is evident that what has been called the B mechanism corresponds to the activity of the posterior lobe of the pituitary gland whose importance in other Amphibia is well established. This conclusion is confirmed by subsequent experiments on the effect of injection. Two hypotheses would account for the new fact which is brought to light in the foregoing table. If the removal of the pituitary gland *as a whole* involves the elimination of the W mechanism it would be expected that the normal animal would, in favourable circumstances, be capable of more extreme pallor than the completely hypophysectomised animal.

The simplest hypothesis would then be that mechanism W is identifiable with the anterior lobe of the gland. Alternatively it may well be that the activity of some other endocrine organ is determined by the presence of the pituitary gland. In this connection it may be mentioned that removal of the pituitary gland in tadpoles is accompanied by involution of the suprarenal cortex (Smith).

That one or other of these hypotheses is correct is strongly confirmed by the behaviour of animals in which the anterior lobe alone was removed. This will be seen from the last series of the foregoing table. Removal of the anterior lobe in *Xenopus* is not so easy to perform as in *Rana*. It adheres by its anterior margin. In consequence it is easy to leave a small portion behind. Animals in which it was seen to have been completely removed remained dark on a white-background for 5 or 6 months after operation. Partial recovery occurred in some individuals. It could then be generally established by *post-mortem* examinations that regeneration had occurred. On the other hand, two animals kept alive for over 2 years after removal remained with their melanophores fully expanded in optimal conditions for pallor (Plate 3). Were it not for the fact that it has been possible to study the reactions of animals surviving for a long period after operation, it might be possible to attribute this result to operative disturbance of the posterior lobe. The long continued survival of the operated animals makes it highly unlikely that such an explanation is valid. It would thus appear that in *Xenopus levis* the white-background response depends upon the presence of the anterior lobe of the pituitary gland and that the black-background response depends upon the presence of the posterior lobe.

It seemed desirable to extend the scope of the investigation to include the effect of removing one or both lobes of the pituitary in eyeless individuals. From the accompanying protocol the results of such experiments are seen to be entirely consonant with the conclusions stated above.

Diffuse Light 17° C.

	Number of animals.	Melanophore index.	
		White- background.	Black- background.
Eyeless (pituitary intact)	21	3.7	—
Eyeless (anterior lobe removed 2 months previously)	21	4.9	—
Eyeless (whole pituitary removed 5 months previously)	21	—	1.7

(d) *Injection into Hypophysectomised Toads.*—Injection of an extract of the posterior lobe of the pituitary gland in *Xenopus* as in *Rana* evokes expansion of the melanophores. Experiments on the effect of injecting pituitary extracts

into normal and hypophysectomised toads revealed a peculiar circumstance. The hypophysectomised animal has a lower threshold and the duration of the darkening produced is much greater than is the case with the normal animal. This conclusion was so unexpected that a long series of experiments were carried out to test it. In some of these commercial extracts were employed. In others extracts prepared in the laboratory from fresh bovine glands were used. The intraperitoneal method was adopted. All animals used in these experiments had been previously kept for a week on a white background. The commercial extract chiefly employed was that known as pitressin (Parke Davis). This is a highly concentrated extract of the pressor substance. The separated oxytocic principle Pitocin has no action on the melanophores. Three representative protocols will be cited.

(1) Fifteen normal and 15 hypophysectomised toads each received a dose equivalent to 0.4 pressor units of Pitressin. For the normal group a maximal effect was reached at about 4 hours with a melanophore index of 4.1. The maximal effect for the hypophysectomised toads was not reached until 6 hours from injection had elapsed, the melanophore index being 4.0. Twelve hours after injection the melanophore index of the hypophysectomised group was 3.1 and of the normal 2.0 (see fig. 6).

(2) To emphasise the difference in threshold a lower dosage was administered

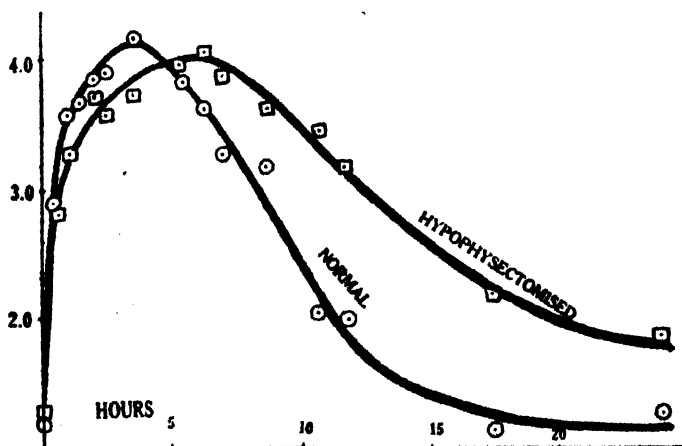


FIG. 6.

in a precisely parallel experiment (fig. 7). In this experiment 0.2 pressor units were injected into each individual. The maximal value of the melanophore index of hypophysectomised group was 3.6. That of the control group

was 2.9. Thirteen hours after injection the hypophysectomised group had reached 3.0, the normal group had come down to 1.7.

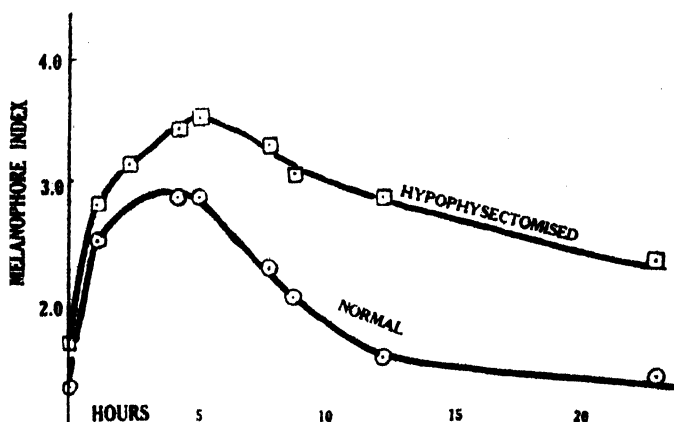


FIG. 7.

There is an obvious objection to any conclusive deduction from the foregoing experiments. The posterior lobe of the pituitary gland has been shown by Krogh and his collaborators to control the state of tone of the capillaries. In this investigation as in Krogh's experiments it was frequently observed that the capillaries of hypophysectomised animals were dilated. It would therefore be expected that injection of pituitary extracts containing the pressor principle would constrict the peripheral vessels of the normal animal to a greater extent than those of the hypophysectomised animal. Constriction of the vessels would naturally tend to mask the effect of the melanophore stimulant B. This ambiguity in the preceding experiments can be eliminated. Hogben and Gordon (1930) have shown that the pressor and melanophore activities of posterior lobe extracts depend on different substances. The pressor activity can be completely destroyed leaving the melanophore activity intact. Indeed the melanophore activity of posterior lobe extracts is increased when the pressor substance is destroyed. This would be expected from the considerations which have been advanced in the foregoing remarks. To place the issue on a more secure foundation experiments were conducted to test the action of extracts, in which the pressor substance had been destroyed.

(3) To an aqueous solution of Pitressin an equal quantity of 2.5 molar sodium hydroxide was added. This was allowed to stand for 3 hours in the cold, neutralised with hydrochloric acid, and diluted to the appropriate strength for

injection. The dose injected into eight normal and eight hypophysectomised toads (females, average weight 72 grams) was equivalent to one-tenth cubic centimetre Pitressin. As will be seen from fig. 8, the melanophore index of the hypophysectomised group 10 hours after injection was 4.0, that of the normal group had come down to 2.0. Twenty-two hours after injection the melanophore index of the hypophysectomised was 2.8.

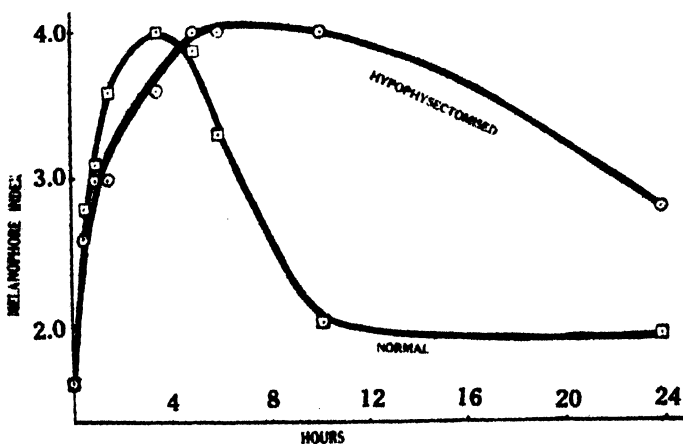


FIG. 8.

The results of the experiments recorded above do not resemble earlier observations on the injection of posterior lobe extracts into normal and hypophysectomised frogs. Hogben and Winton (1923) found that the minimal dose for hypophysectomised frogs was higher than for normal ones. Several explanations of this discrepancy might be advanced. One is that in Hogben and Winton's experiments animals were injected soon after operation. All the animals employed in the foregoing experiments had been living in the laboratory for at least 9 months after operation. There seems to be only one apparent explanation of the greater sensitivity of *Xenopus* to pituitary extracts after its pituitary gland has been removed. If the removal of the anterior lobe of the pituitary gland eliminates the W mechanism which has been postulated in Part II of this communication, it is easy to see why this result should occur. In the hypophysectomised animal the B substance injected into the circulation is not antagonised by the presence of W substance in excess. On the other hand, it is not reinforced by the B substance already present in the normal animal. Thus the hypotheses that two endocrine agencies are involved in the colour responses of *Xenopus laevis* is fully compatible both with the observations on the effects of injection of pituitary extract on normal and hypophysectomised

frogs as recorded by Hogben and Winton, and with the contrary result obtained in experiments on *Xenopus laevis* here described. In any case the peculiar fact that the completely hypophysectomised clawed toad has a greater rather than a lower sensitivity to posterior lobe extracts as compared with the normal animal reinforces from an unexpected source the conclusion that the W mechanism is closely associated in some way with a part of the pituitary gland and almost certainly not the same part as that which is identifiable with the B mechanism.

(e) *The Morphology of the Pituitary Gland of Xenopus.*—Experiments on *Rana fuscigula* cited in Part I show that a double endocrine mechanism also exists in *Rana* as well as in *Xenopus*. Hogben and Winton did not find that removal of the anterior lobe alone resulted in permanent darkening of the skin in their experiments on *Rana temporaria*. We have removed the anterior lobe from 30 individuals of *Rana fuscigula*. All showed a generalised condition of melanophore expansion for several days after operation. They eventually regained their power to exhibit the reaction of pallor to white-background. We were thus led to enquire whether there exists any morphological difference between the anterior lobe of *Xenopus* and *Rana*.

The anterior lobe of the frog can be easily detached from the rest of the complex both by a fine jet of fluid or gentle lateral pressure with a needle. That of *Xenopus* obstinately clings to the stalk by a tapering process inserted into the cleft extremity of the *tuber cinereum*. This process which is labelled as the basophil area in the accompanying figures (fig. 9) and photomicrographs (Plate 4) is histologically differentiated from the rest of the anterior lobe by its lobular structure, highly vascularised character and freedom from oxyphil cells. In *Rana* and *pars tuberalis* is represented by paired plaques of glandular tissue which are situated on the *tuber cinereum* quite separately from the rest of the gland. It has been shown by Atwell (1919) that these paired structures are absent in Urodeles. The anterior lobe is continued forward over the *tuber cinereum* as a pair of processes composed of basophil cells which are not actually separated from the *pars anterior* proper. Atwell and De Beer identify this continuation of the anterior lobe over the *tuber cinereum* in Urodeles with the *pars tuberalis*. Our own sections led us to conclude that the pituitary of *Xenopus* is of the salamandrine type.

The hypothesis that the W mechanism is located in the *pars tuberalis* would accommodate all the discrepancies discussed in the preceding pages. The operation of hypophysectomy devised by Hogben removes the *pars anterior*, *pars intermedia* and *pars nervosa*, but not the *pars tuberalis* in *Rana*. If we

are right in believing that the pituitary of *Xenopus* is of the Urodele type, hypophysectomy in *Xenopus* involves in addition the removal of the *pars*

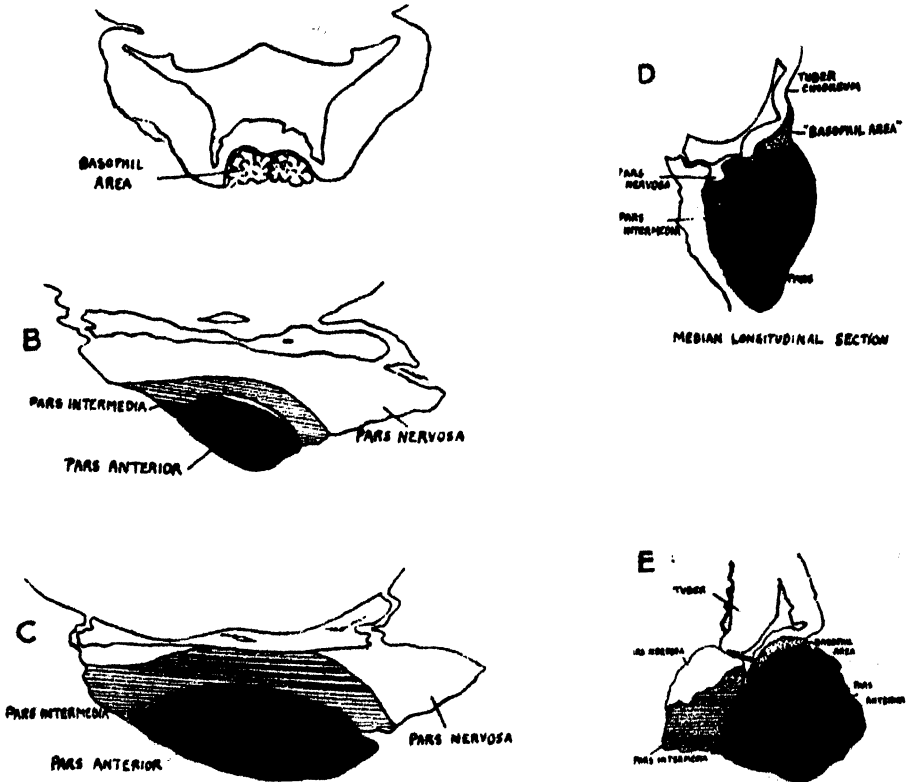


FIG. 9.—Transverse sections (plane at right angle to long axis of the brain) of the pituitary body of *Xenopus*—A through anterior extremity, B middle, C posterior. D and E—Longitudinal sections of the pituitary of *Xenopus* in vertical and tangential planes.

tuberalis. We know that the B substance is located in the *pars intermedia*. If the W substance is located in the *pars tuberalis* the circulation of a hypophysectomised *Rana* contains the W but not the B substance, while that of the completely hypophysectomised *Xenopus* contains neither B nor W.

Injection of boiled acid or alkaline extracts of ox anterior lobe does not induce pallor in *Xenopus*. The injection of boiled acid extracts of the stalk of the ox pituitary, on the other hand, into dark animals was always found to produce a drop in the melanophore index and in some cases to induce macroscopic pallor. This result was never obtained with extracts of brain tissue, but we hesitate to make the definite statement that the *pars tuberalis* of the ox contains a specific substance which promotes the contraction of amphibian

melanophores, because we have been unable to obtain macroscopic pallor in all individuals of a batch injected at any one time with extracts of this material.

It may be well to recapitulate the argument developed in this section in more concise form. Removal of both lobes of the pituitary gland in *Rana* leads to permanent maximal pallor. In *Xenopus* it leads to permanent pallor but not to maximal pallor. Removal of the anterior lobe alone leads to permanent and maximal expansion of the melanophores of *Xenopus*. This result does not occur in *Rana*. After removal of both lobes *Xenopus* is more sensitive to the B substance, whereas *Rana* is less sensitive to the B substance. If W is associated with the *pars tuberalis* removal of both lobes in the frog would result in removal of B without removal of W, while removal of both lobes in *Xenopus* would result in removal of B and W. If W is associated with the *pars tuberalis*, removal of the anterior lobe in *Xenopus* would involve removal of W without removal of B, while in *Rana* it would involve removal of neither B nor W. If W is associated with the *pars tuberalis* a hypophysectomised frog having no B will have a higher threshold for the B substance because W is still present in the circulation ; whereas *Xenopus* might have a lower threshold after removal of both lobes, because the circulation contains no W to antagonise the action of B.

Summary of Part III.

(1) *Xenopus laevis* can be kept alive indefinitely after removal of the whole pituitary (complete hypophysectomy) or of the anterior lobe alone. Such animals have been kept under observation in the laboratory for a period of a little more than 2 years.

(2) Removal of the whole gland abolishes the black-background response.

(3) Removal of "anterior lobe" alone abolishes the white-background response in *Xenopus* but not in *Rana fuscigula*.

(4) The difference in behaviour of *Xenopus* and *Rana* is correlated with a structural difference in the pituitary gland. In *Xenopus* the *pars tuberalis* is included in the anterior lobe. In *Rana* the *pars anterior* and *pars tuberalis* are quite separate. It is therefore suggested that the W substance is contained in the *pars tuberalis*.

(5) Normal animals after prolonged exposure to a white-background are slightly paler than completely hypophysectomised animals in the same situation. This is explicable on the assumption that the W mechanism is directly or indirectly dependent on the activity of the "anterior lobe" as suggested by (3).

(6) Injection of posterior lobe extracts into completely hypophysectomised

clawed toads produces darkening as in frogs. The removal of the whole gland renders the animal more sensitive and not less sensitive to the injection of pituitary extracts. This could be explained on the assumption that B substance introduced into circulation is not antagonised by the presence of W, if the W mechanism is dependent in some way on the anterior lobe of the pituitary gland as indicated in (3) and (4).

(7) The primary response is independent of the pituitary gland.

*Part IV.—The Relation of the Nervous System to Colour Change in
Xenopus laevis.*

The data presented in Part II make it well nigh impossible to entertain the view that the pituitary secretion is a static condition essential to the proper exercise of a dynamic nervous control.

It is now proposed to bring forward direct evidence (a) to show that the secondary or eye-co-ordinated response of *Xenopus* can be brought into play independently of efferent nerves supplying the skin; and (b) to determine whether the nerve supply of the skin plays a significant rôle in the primary response.

(a) Relation of the Nervous System to the Primary Response.

In striking contrast to the slow development and subsidence of the secondary and co-ordinated photic responses for which the eye is the receptor organ the primary response, which is best studied in the eyeless animal, occupies between 30 minutes and three-quarters of an hour. From the time relations of the primary response we have nothing to guide us in deciding, whether it is (a) a simple reflex, (b) partly reflex and partly endocrine, or (c) a direct reaction of the melanophores themselves to light. At first sight the last hypothesis seems the most likely. On the other hand, it has been shown by Parker (1904) that there are photoreceptors in the skin of Amphibia, since eyeless frogs show phototropic behaviour. Unpublished experiments by Zoond in this laboratory prove that this conclusion applies to *Xenopus laevis*. The second hypothesis cannot be excluded conclusively, but the experiments recorded in the preceding section eliminate the pituitary from any part in the primary response. It has been shown that the primary response is manifest in the completely hypophysectomised animal. It is now necessary to decide between the first and the last hypotheses.

If there is a peripheral nerve supply to the melanophores of *Xenopus*, a simple reflex depending upon photoreceptors in the skin might be the explana-

tion of the primary response. To test this the spinal cord was completely destroyed in 24 animals. The temperature of the room was 19° C. The webs were spread out and 12 animals were placed in darkness, the others being placed in white containers in bright light. At the end of 1 hour the conditions of the webs of the two series was recorded, the figures being :—

Darkness	2.1 ± 0.14
Light	2.4 ± 0.14.

The two series were then reversed and a second record taken after 2 hours. This resulted in the following data :—

Darkness	1.9 ± 0.14
Light	2.25 ± 0.19.

The consistency of this result seems to yield fairly conclusive evidence in favour of the view that the primary response is completely independent of the C.N.S. as well as of the pituitary. This amplifies a previous statement that the primary response of *Xenopus* is essentially like the Reptilian response to light. The photic response of Reptiles is due to direct action of the light on the melanophores without the intervention of any system of co-ordination.

(b) Effects of Nerve Section on the Secondary Response.

The precautions necessary in the interpretation of experiments on nerve section have been mentioned. An experimental precaution too often overlooked by previous workers is the desirability of working with animals that have completely recovered from operative disturbances. In selecting *Xenopus* for a more extensive investigation of the control of Amphibian colour change, benefit has been derived from the experience of Professor Jolly, whose work has shown that *Xenopus* is particularly well suited for survival experiment involving section of nerve trunks. Various types of experiment were carried out. Three only will be described in detail.

(i) *Section of the Optic Nerve.*—In view of the recent work of Perkins (1928) on Crustacean chromatophores it seemed worth while to compare the effects of section of the optic nerve with the effects of total removal of the eyes. Perkins has published an account of experiments which suggest that the crustacean eye contains secretory elements which exert a hormonal influence on colour change. The results of experiments undertaken with this end in view indicate that the eye exercises its influence on colour response in *Xenopus*

lavis exclusively through impulses initiated in the retina and transmitted by the optic nerves to the brain.

	After 12 weeks on black-background.	After 5 weeks on white-background.
Optic nerves cut at chiasma (11 toads)	3.7 $\pm$ 0.3	3.7 0.1
Eyeless (14 toads)		3.5 0.2

The above record was taken at 15° C. in diffuse light. Aqueous extracts of the eye did not yield results analogous to those recorded by Perkins.

(ii) *Section of the Sciatic Plexus*.—The entire sciatic plexus of one side (left) was destroyed in six toads. After 3 weeks the incision had healed and the circulation of the web was not noticeably deranged on the operated side. The following readings were taken, the initial time being 3 weeks after operation. Where readings were taken at midnight the fact has been noted, as demonstrating the “diurnal variation” elsewhere described.

Time.	Left leg (paralysed).	Right leg (normal).
0.00	2.16	2.16
Transferred to black-background (0.00)		
2.30	3.82	3.82
5.00	4.33	4.33
13.00 (midnight)	4.0	4.0
24.30	4.5	4.5
Transferred to white-background (24.30)		
32.00	2.0	2.0
37.00 (midnight)	1.6	1.6
50.00	2.0	2.0
61.30 (midnight)	1.5	1.5
76.30	1.6	1.6
Transferred to black-background (76.30)		
81.30	3.7	3.7
120.00	4.8	4.8
Transferred to white-background (120.00)		
216.00	1.3	1.3
Transferred to black-background (216.00)		
276.00	4.0	4.0
288.00	4.16	4.16

From these data it is seen that there is complete agreement between the behaviour of the two limbs even as regards the diurnal variation, which at first sight suggests a conditioned reflex. To make certain that the limbs react

at the same rate further records were taken at shorter intervals 6 weeks after operation.

Time.	On white-background.	
	Right leg.	Left (operated) leg.
0·00	1·5	1·5
	Transferred to black-background	
1·00	3·0	3·0
3·00	3·8	3·8
4·30	4·0	4·0
9·30 (midnight)	3·8	3·8
19·30	4·3	4·5
22·30	4·6	4·6
31·30 (nearly midnight)	4·0	4·0
	Six days later on black-background	
0·00	4·0	4·0
	Transferred to white-background	
6·00	2·2	2·2

Again the diurnal variation is well shown in both legs, and there is no discrepancy as regards the rate of reaction. This is not surprising, since the circulation in the operated limb seemed to be restored to its normal condition macroscopically. From this it appears that the diurnal variation, if a conditioned reflex, is a conditioned reflex of the secretory activity of the pituitary gland.

(iii) *Section of the Spinal Cord.*—It is an easy matter to cut the spinal cord of *Xenopus* at the level of the third spinal nerve and obtain a spinal preparation which will survive for some months, if kept in a moist container. At a higher level spinal transection is extremely difficult to execute without fatal consequences within a few days after operation. We have been able to sever the cord above the level where the first spinal nerve emerges, and keep the operated animals alive sufficiently long to show that this operation is compatible with the complete exercise of the "background" reaction. The time relations of colour change are invariably modified. This might well be expected from the ensuing vasomotor disturbance. Typical protocols are as follows, the temperature being 15 to 17° C.

(a) Section of the spinal cord between the occiput and the atlas vertebra ; and anterior to the first spinal nerve.

	Days after operation.	Melanophore index.
Transferred to black-background..	15	1.0
	16	2.5
	17	3.0
Transferred to white-background..	18	4.0
	19	2.0
Transferred to black-background..	21	2.0
	23	4.5
Transferred to white-background..	24	4.5
Transferred to black-background..	26	1.5
	27	3.5
	28	4.0
	29 (autopsy)	—

(b) Same level as (a).

	Days after operation.	Melanophore index.
Transferred to black-background.....	16	1.0
	18	3.0
Transferred to white-background.....	19	4.0
	19 (4 hours later)	1.0
Transferred to black-background.....	21	1.0
	23	2.0
	24 (autopsy)	—

(c) Same as (a) and (b).

	Days after operation.	Melanophore index.
Transferred to black-background.....	5	2.0
	7	2.0
Transferred to white-background.....	9	4.5
	10	2.0
	11 (autopsy)	—

(d) Section of the spinal cord between the axis and atlas, but also in front of the first spinal nerve.

	Days after operation.	Melanophore index.
Transferred to black-background..	10	2.0
Transferred to white-background..	14	4.5
Transferred to white-background..	15	1.5
	16	3.0
	17 (autopsy)	—

In the next series protocols of animals in which transection at lower levels was carried out will be included.

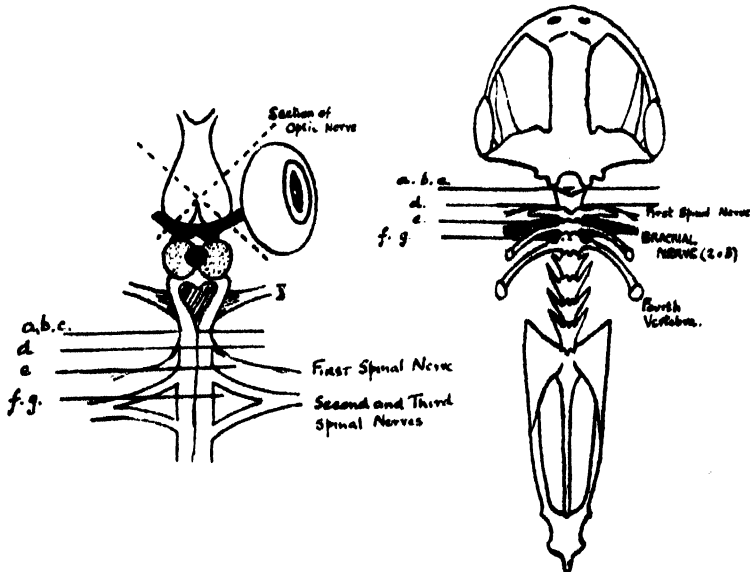


FIG. 10.—Diagrams to show levels of transection in experiments recorded in text.

(e) Section of the spinal cord between the first and second spinal nerves.

	Days after operation.	Melanophore index.
Transferred to black-background..	10	1.0
Transferred to white-background..	19	4.5
Transferred to black-background..	20	1.5
	21	3.0
	22	3.5
	23 (autopsy)	

(f) Section of the spinal cord between the second and third spinal nerve.

	Days after operation.	Melanophore index.
Transferred to black-background.....	15	1.5
Transferred to white-background.....	16	5.0
Transferred to black-background.....	17	2.0
Transferred to white-background.....	18	5.0
	19	2.5
	20	2.0
	21 (autopsy)	—

(g) Same level as (f).

	Days after operation.	Melanophore index.
Transferred to black-background..	10	1.0
Transferred to white-background..	14	4.5
	15	3.0
	21	2.0
	24 (autopsy)	

(h) Three animals survived the operation of transection of the cord at the level of the 7th, 8th and 9th vertebræ together with section of the sympathetic chain. Their behaviour was uniform, and it is therefore permissible to give the figure in averages. The time is here reckoned from 3 weeks after operation.

	Time (hours).	Melanophore index.
Transferred to white-background..	0.00	4.3
	2.30	2.0
	5.0	1.7
	13.00 (midnight)	1.7
	24.30	1.7
	32.00	1.3
	37.00 (midnight)	1.3
	50.00	1.7
	61.30 (midnight)	1.3
Transferred to black-background..	76.30	1.6
	81.30	3.0
	120.00	3.7
Transferred to white-background.	216.00	4.0
	276.00	3.3
	288.00	1.3

The exact definition of the level at which section of the cord was carried out was made by *post-mortem* examination. The neuromuscular reflexes of the operated animals give some indication. Pressure on the tips of the digits of the forelimbs evokes withdrawal of the hind leg in toads in which the cord is transected in front of the emergence of the third spinal nerve. Blinking of the eye to the same stimulus occurs if the section is posterior to the second nerve. In animals in which the section has been made between the second and third nerves, which together form the brachial plexus, reflex movement in both caudad and cephalad extremities can be elicited. Only animals in which a corneal reflex could be elicited were kept for observation.

The foregoing experiments confirm the previous statement that the diurnal variation (seen in (h)) is independent of the peripheral nerves. Two features call for special comment. The first is that the black-background response of

spinal animals is abnormally sluggish. The second is that the black-background response rarely proceeds to the limit of completion reached in normal toads, though macroscopically the operated animals were indistinguishable from normal ones. The sluggishness can only be interpreted in terms of circulatory disturbance which was evident from the dilation of the blood vessels.

The animals referred to as (b) and (h) never showed the extreme expansion (5.0) which is characteristic of the normal animal on a black-background. But the white-background response which is more rapid than in normal animals (see especially (b)) was complete. In observing the responses of animals in which the C.N.S. was cut at the level of the 1st, 2nd and 3rd vertebræ, it was noted that in a few animals a complete macroscopic reaction to white background by pallor did not occur. The melanophore index varied between 5 and 2.5 or 3.0 when the animals were transferred from black- to white-background and *vice versa*. These animals which never became completely pale in outward appearance gave the complete response to black-background. The last protocol to be cited refers to an individual of this type.

(j) Section of the spinal cord between 2nd and 3rd vertebræ.

	Days after operation.	Melanophore index.
Transferred to black-background	27	2.5
Transferred to white-background	28	5.0
Transferred to black-background	29	2.5
	31 (autopsy)	5.0

It has been remarked that the operation was accompanied by a considerable general disturbance of the circulatory system as indicated by the state of the capillaries in the web. Where a double endocrine mechanism exists, it is impossible to predict whether a generalised condition of capillary dilation would reinforce the black-background or the white-background response. Whatever is the true explanation, the incomplete responses of some operated animals after spinal transection certainly cannot be used to support the view that the melanophores of *Xenopus* are directly innervated like those of Amphibia and Fishes. Nerve section and spinal transection in Fishes and Reptiles are accompanied by purely segmental disturbances. The segmental phenomena have been recorded photographically by Hogben and Mirvish (1927). Even if it had not been shown that some animals will go through the entire range of colour response after complete spinal transection in front of the first spinal

nerve, such generalised disturbances as those to which we have directed attention, could be quoted with more cogency as an argument against rather than an argument in favour of the hypothesis that the melanophores of Amphibia are directly innervated. The foregoing experiments do not prove that Amphibian melanophores have no peripheral nerve connections. They do show, however, that the phenomena of colour change can be interpreted without recourse to such an assumption; and they provide crucial evidence for the view that the secretory activity of the pituitary gland fluctuates with external conditions, and is not merely a static condition for the exercise of a direct peripheral nervous control over the pigmentary effector system.

(c) *Discussion.*

In a recent communication Kropp (1928) has reopened the question of nervous control of the pigmentary effector system of Amphibia. He describes experiments which he regards as incompatible with a conclusion stated elsewhere by Hogben (1924). This is that "the regulation of colour response by fluctuating pituitary secretion is adequate to interpret all the accredited phenomena in Amphibia." He appears also to imply that Hogben denies the existence of nervous control of the pigmentary effector system. Dr. Kropp's communication contains, with one exception to be mentioned later, no new data bearing on this problem. It seems desirable to reaffirm somewhat more explicitly the position previously adopted by one of us and confirmed by the results of this enquiry.

The independent work of P. E. Smith, Hogben, Hogben and Winton, Krogh and his pupils, Houssay and others has now established beyond doubt the fact that the pituitary gland is essential to the rhythm of colour change in adult and larval Amphibia. The fact that the eyes are the receptors for the "secondary" light reaction is also established. A large literature dealing with data bearing on the supposed direct innervation of Amphibian melanophores reveals complete disagreement between the testimony of different workers with regard to the most vital points. The direct evidence of Krogh and Houssay has shown that the quantity of circulating hormone is rarely if ever in normal circumstances much below the necessary threshold for expansion. Given the fact that pituitary secretion is necessary to the expansion of Amphibian melanophores, it follows that reducing the blood supply of a region of the body, when the melanophores are expanded, may evoke contraction, while increasing it in the pale animal may call forth expansion of the melanophores of the part affected. Since the stimulation of any nerve

in the vertebrate body generally evokes vasoconstriction, while section of nerves is usually followed by dilatation, no experiments can conclusively prove the direct innervation of the melanophores unless the effects of stimulating or of excluding the influence of vasoconstrictor fibres is eliminated.

Kropp's paper, like the earlier work of Hooker, gives ample illustration of the importance of vasomotor effects in the interpretation of his results. After section of the sciatic nerve he finds that "for 2 days after the operation the operated leg reacts normally *except for a slight lag in time*" (p. 293, italics inserted). On p. 297 he notes that after section of the sciatic the operated leg did not become as pale as its fellow after injection of adrenaline into the circulation. On p. 304 he states that "no change was observed following simple transection of the sciatic plexus . . . synchronous colour changes occurred with only a slight lag comparable in every way to that which followed cutting the sciatic nerve." On p. 306, after describing the effect of spinal transection in the tail of the tadpole, he notes that the expansion of the melanophores "is not permanent as in *Fundulus*," which has an undoubted innervation of the pigmentary effector system.

All the foregoing experiments provide suggestive indications that the phenomena with which Kropp was dealing are capable of quite a different interpretation from that which he advocates. The line of evidence upon which he chiefly relies is derived from stimulation of the sciatic plexus and its associated sympathetic components. It is difficult to follow the reasoning on p. 306 where Kropp states that "these cases show conclusively that co-ordination of the melanophores occurs through sympathetic fibres." In these experiments the sciatic nerve alone, the sympathetic roots associated therewith alone, and the entire plexus were stimulated. In all cases some degree of pallor was obtained. When the entire plexus was stimulated the maximal effect occurred. The pigment cells showed "a very disorganised appearance" and "they appeared to be broken." There is nothing to indicate that this effect was a reversible one. On what grounds does Kropp assume that these are not vasomotor effects? He tells us, "when circulatory disturbance was unavoidable extreme care was exercised with the control leg. Barring the actual stimulation or cutting the control leg was subjected to the same operations and conditions as the experimental leg." From this it would appear that Kropp has entirely failed to appreciate the fundamental issue. It is impossible to cut or stimulate any peripheral trunk without affecting its vasomotor components. The procedure which Kropp adopted could not possibly avoid the intervention of circulatory disturbances. These were uncontrolled,

however great the care adopted to subject the "control" leg to similar treatment. Curiously Kropp gives no indication of having made personal observations on the dilatation of the capillaries of the web such as have been recorded by Krogh and by Hogben (1924). Had he done so he would have obtained direct evidence of the ubiquitous presence of vasomotor components in peripheral nerves.

Two minor points in Kropp's communication demand brief comment. Referring to observations on the retinal pigment cells he states "it is remarkable that the melanophores of the operated eye do not expand with those of the control eye, if the adaptation to darkness is co-ordinated through the presence of pituitrin in the blood." As a matter of fact, Hogben and Winton have recorded the observation that the retinal pigment cells do not react to pituitary extract in the same way as the melanophores of the skin. This criticism has therefore no evident bearing on the question. The second point arises in connection with the following quotation, which suffices to show that Kropp has not read with great care the series of papers which he cites in his literature list, and more especially one in which Hogben deals specifically with every issue raised in his paper (Pigmentary Effector System—IV). "In the frog," he writes on p. 312, "stimulation with a weak faradic current of the cloaca, lips, surface of the mouth and nictitating membrane of the eye, causes blanching over the entire animal within 5 minutes. These results are in entire accord with the conclusions of Hogben (1924) regarding melanophore contraction under similar conditions, and, like Hogben, I conclude that this type of co-ordinated melanophore reaction is definitely brought about by means of adrenalin secreted into the blood stream." There are three comments which it seems appropriate to make in this connection. The first is that Hogben has never recorded melanophore contraction under similar conditions. The second is that it certainly does not occur in *Rana fuscigula*, or in *Xenopus laevis*, the only two species of Amphibia that have been accessible for the present investigation; nor, it would seem, does it occur in *Rana temporaria*, the species on which the work of Hogben and Winton and of Hogben was based. The third is that in none of his publications has Hogben expressed, or apparently entertained, the conclusion that adrenal secretion plays any significant part in the regulation of the chromatic function in Amphibia. On the other hand, if Dr. Kropp's observation were correct, it should provide him with a critical experiment which would be adequate to settle the question. In the chameleon stimulation of the cloaca does *reversibly* produce generalised pallor within a few minutes both in the intact animal, and in the posterior severed portion

of the trunk after evisceration, so that all circulatory effects are excluded. Hogben and Mirvish (1928) have recorded this phenomenon in the chameleon, with photographic reproduction of a typical experiment. Photographs of similar phenomena in the frog would go far to disposing of any remaining doubt as to the direct innervation of Amphibian melanophores. Till such are produced, it suffices to reaffirm the conclusion stated by Hogben (p. 265, *loc. cit.*): "it is not easy to prove a negative, and to deny that Amphibian melanophores are subject to direct innervation would not be justified, but in the light of the new evidence one can approach the question without a strong predisposition in favour of the positive attitude."

Summary of Part IV.

- (1) The section of the entire nerve supply of the hind limb in *Xenopus* does not interfere with normal colour response to photic stimuli.
- (2) Spinal transection in front of the first spinal nerve is compatible with a complete "background" reaction in the melanophores of the hind limb.
- (3) At no level is spinal transection accompanied by segmental chromatic changes.
- (4) Section of the optic nerves at the chiasma evokes the same result as removal of the eyes.
- (5) The primary response is not dependent on the C.N.S. It appears to be a direct reaction of the melanophores to incident illumination.
- (6) The diurnal rhythm is not dependent on the peripheral nerve supply of the skin.
- (7) There is no need to assume that the nervous system plays any part in Amphibian colour change other than in transmitting the stimulus from the eye to the pituitary gland.

Part V.—General Conclusions.

The experimental data have already been summarised at the end of each section. A brief statement of the main conclusions which have emerged from this investigation will therefore suffice.

- (1) Two separate endocrine agencies co-operate in the co-ordination of colour change in *Xenopus laevis*. This conclusion is sustained by several quite independent lines of evidence. Its cogency has become increasingly strengthened as the investigation developed. The three most important reasons for advancing the existence of two distinct endocrine agencies are (a)

the "intermediate" condition of the melanophores of the eyeless animal interpreted in the light of the observations recorded in Part IV ; (b) the analysis of the time relations of the visual response set forth in Part II ; (c) a study of the threshold of normal and hypophysectomised individuals injected with pituitary extracts containing the B substance or "melanophore stimulant" of earlier communications in this series. That this type of co-ordination is generally found in Amphibia has not been proved conclusively. The observations of Laurens on *Urodele* larvæ together with our own, at present unpublished studies on *Rana fuscigula*, strongly support the view that *Xenopus* is a peculiarly favourable species for demonstrating a characteristic of the chromatic function common to other Amphibia.

(2) Granting the conclusion stated in the preceding paragraph, it will be asked whether the two agencies involved in the chemical co-ordination of the visual response can be defined in more precise terms. One of these which has been referred to as the B substance is involved in the black-"background" response. Evidence submitted in Part III sustains the conclusion that substance B is the "melanophore stimulant" which has been made the subject of previous investigations by Hogben and Winton. The B substance appears to be manufactured pre-eminently by the *pars intermedia*. As shown by Dreyer and Clark (1924), Fenn (1924) and by Schlapp (1925) it is not identical with the oxytocic autacoid. As shown by Hogben and Gordon (1929), it is not identical with the pressor substance. The identification of the W substance which is concerned with the "white-background" response raises an entirely new issue, already adumbrated in an earlier communication (Hogben, 1924) in which it was pointed out that "the paradoxical results of blinding in young axolotls might" be interpreted "by supposing that colour response in *Urodeles* depends upon a balance between pituitary activity and some other mechanism . . .". The new information presented in Part III of this communication clearly shows that the W substance is associated, directly or indirectly, with the activity of some component of the pituitary gland itself. The discordant results of operative removal of the whole gland or a part of it in *Xenopus* as compared with *Rana* are capable of being interpreted upon the assumption that the W substance is manufactured by the *pars tuberalis* or by some endocrine organ which is influenced by the activity of the *pars tuberalis* of the pituitary gland. This conclusion rests upon definite morphological differences between the pituitary complex of *Rana* and that of *Xenopus*. Since the observations recorded in Part III were completed, we have sent to Dr. de Beer fixed material and slides of the brain and hypophysis of *Xenopus* for confirmation of our own

findings. Dr. de Beer, who has made an extensive study of the comparative morphology of the pituitary gland, has kindly examined our material and compared it with his own preparations of *Rana* and *Triton*. In a private communication he reports that he is entirely in agreement with our conclusion that the pituitary gland of *Xenopus* is essentially of the Urodele rather than the Anuran type.

In probing the identity of substance W, it may be pointed out that the evidence presented in Part IV narrows the field. From the results of spinal transection in *Xenopus* and earlier observations of Rogers on *Diemyscelus* it is quite clear that W is referable—directly or indirectly—to some gland located in the head extremity. This, in effect, leaves only two likely possibilities, namely, some constituent of the pituitary gland or the pineal body. It would not be justifiable to dismiss the latter from consideration, especially in view of the fact that McCord and Allen (1917), Huxley and Hogben (1923) and Swingle (1922) have shown that extracts of the mammalian pineal contain a specific autacoid which is capable of evoking the white background response.

3. Hitherto it has seemed that the external agencies affecting pigmentary effector activity, the receptive structures involved in recording these changes and the co-ordinating processes involved are fundamentally dissimilar in Reptiles and Amphibia. In the light of new evidence presented in this communication this conclusion may require modification. It is now seen that the relation of Amphibian colour change to light is complex, involving a primary response of the melanophores themselves and a co-ordinated response for which the eye is the receptor organ. The former, though of very subordinate importance, is essentially identical with the familiar response of the chameleon and *Phrynosoma*. In our own observations on the chameleon no conclusive evidence of a visual response was obtained; but this may well be because it is only possible to observe gross macroscopic changes. Redfield did find evidence of what he calls an adaptive response after prolonged exposure of *Phrynosoma* to light and dark backgrounds. It may well be that the diverse phenomena encountered in different species of Amphibia and Reptiles are due to the relative predominance of different systems of response rather than to a fundamental discontinuity in the character of pigmentary effector activity.

Summary.

(1) Colour change among Amphibia in response to photic stimuli involves two components. One is independent of the eyes, of the nervous system or the pituitary gland. The melanophores possess the property of reacting to

light directly by expansion. This *primary* response is predominant in a few Amphibia (such as *Necturus*). In *Xenopus*, in adult frogs and in *Amblystoma* larvae it is of subordinate importance. In addition to the primary response there is a *secondary* or co-ordinated response for which the eye is the receptor organ. The visual response involves generalised pallor when the field of vision is occupied by a light-scattering surface, and generalised darkening of the skin when the field of vision is occupied by a light-absorbing surface.

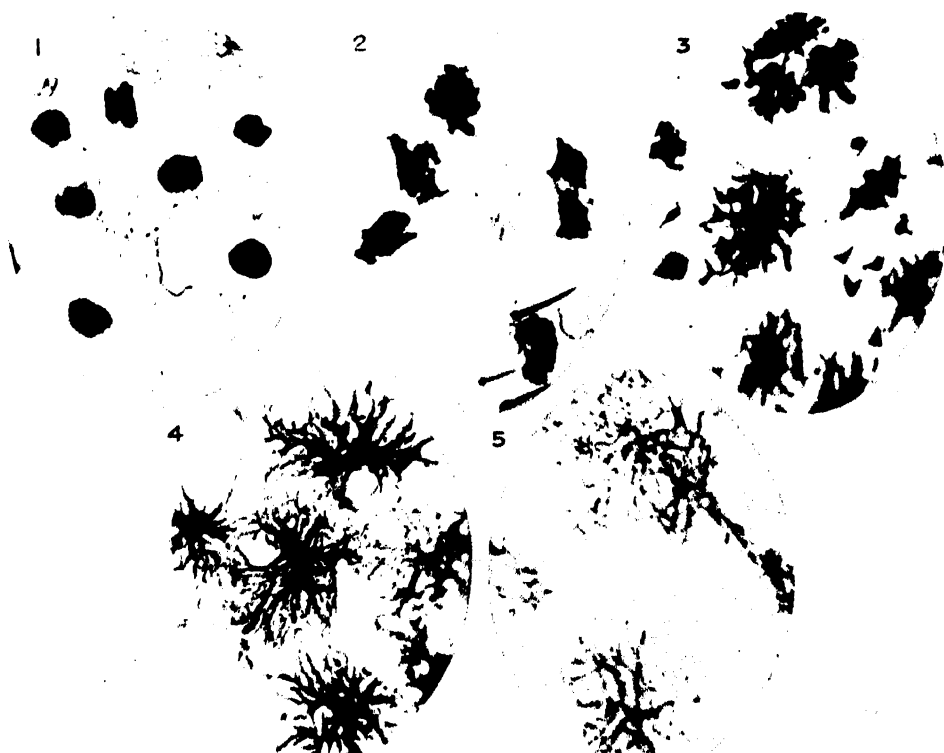
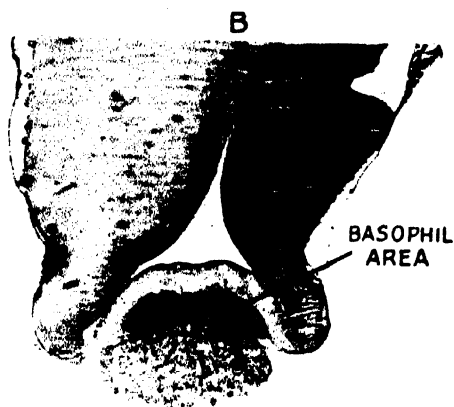
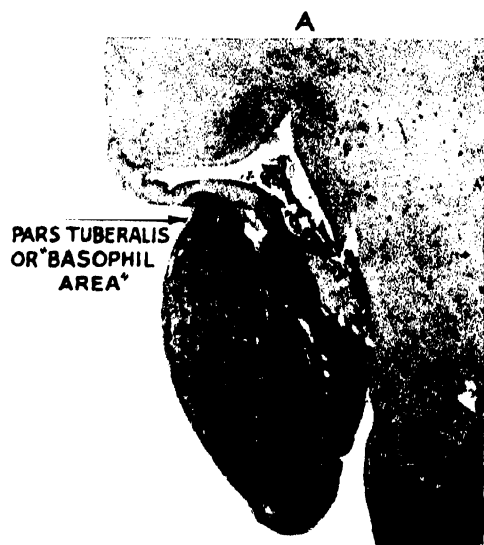
(2) The removal of the eyes abolishes the secondary response. Eyeless animals and animals kept in darkness display neither maximal pallor nor maximal expansion of the melanophores. They are darker than pale animals and slightly paler than dark animals, even in bright light.

(3) The visual response develops more slowly than the primary response. From its time relations it can be inferred that it involves endocrine co-ordination and that two distinct endocrine agencies are respectively involved in the white- and black-“background” responses. The visual response is not abolished by spinal transection at any level.

(4) In *Xenopus* removal of the “anterior lobe” abolishes the white-“background” response. Removal of both lobes, as in *Rana* and in *Urodeles*, abolishes the black-“background” response as well. In *Rana* removal of the anterior lobe does not abolish the white-“background” response. In *Xenopus* removal of the anterior lobe involves removal of the *pars tuberalis* as well as the *pars anterior*. In *Rana* removal of the anterior lobe involves removal of the *pars anterior* alone. From this it would appear that the second endocrine agency, whose existence has been inferred above, is associated directly or indirectly with the *pars tuberalis*.

(5) This conclusion is confirmed by two other discrepancies between observations made upon *Xenopus* and *Rana* respectively. After removal of both lobes a condition of maximal pallor ensues in *Rana* and the sensitivity of the animal to the injection of posterior lobe extracts is diminished. The pallor that ensues after removal of both lobes in *Xenopus* is less pronounced than in *Rana*; and sensitivity to the action of posterior lobe extracts is increased. It is suggested that these discrepancies are explained by the fact that removal of the posterior and anterior lobe of *Rana* does not involve removal of the *pars tuberalis*.





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EXPLANATION OF PLATES.

PLATE 3.

- FIG. 1.—On the left, (a) animal kept in black container for twenty-five months after total removal of the pituitary gland; on the right, (b) normal animal kept in black container for three weeks.
 FIG. 2.—On the left, (a) animal kept on white "background" for twenty months after removal of the anterior lobe of the pituitary gland; on the right, (b) normal animal after three days on a white background.

PLATE 4.

- FIG. A.—Photomicrograph of the pituitary of *Xenopus laevis* in same plate as text-figure 9, D.
 FIG. B.—Tangential section through the foremost part of the "anterior lobe" showing the basophil area clearly differentiated.
 FIGS. 1-5.—Photomicrographs of the melanophores of the web of *Xenopus laevis* in five stages corresponding as far as possible with scale indicated in text-figure 1.

The Ureides of p-Aminophenylstibinic Acid.

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1. Indian kala-azar is a widespread disease which ran a fatal course, uninfluenced by drugs, until it was found that it could be cured by tartar emetic (Di Cristina and Caronia, 1915; Rogers, 1915; Muir, 1915). More recently it has been shown by a number of workers that certain organic antimony compounds can be used with more advantage. These are far less toxic in proportion to their curative power, and are thus suitable for intensive administration. Several of the more important are simple derivatives of *p*-aminophenylstibinic acid (stibanilic acid), of known constitution, for example, the sodium salts of the glucosyl-(stibamine glucoside) and the 3-chloro-4-acetyl-derivatives, also the diethylamine salt of the acid itself. Apart from these, the most interesting is a material prepared by heating stibanilic acid with urea solution, introduced by Brahmachari (1922) under the name "urea stibamine," the nature of which has been the subject of conflicting opinions by other workers as well as by Brahmachari himself. As this substance is in extended use in India, and it is important from the point of view of progress in chemotherapy that the constitution of useful drugs should not remain undetermined, the present investigation, with the initial object of elucidating this, was undertaken.

2. At the outset the product was found to be, not ammonium *p*-carbamido-phenylstibinate, which is Brahmachari's latest view (1924), but a somewhat complicated mixture of colloids. Only partial separation of its constituents could be effected by chemical means, which, however, sufficed to show that this ammonium salt could not be present to any considerable extent, and to indicate the presence of the essential active substance with respect to mouse trypanosomiasis (*Trypanosoma equiperdum*), *s*-diphenylcarbamide-4:4'-

* W. H. Gray is responsible for the chemical section, and J. W. Trevan, H. W. Bainbridge and A. P. Attwood, by whom all the experiments on animals were made, for the physiological.

distibinic acid. Verification of this was afforded by preparation of these two ureides by separate and simpler methods and comparison of their physiological properties with those of "urea stibamine (Brahmachari)."

3. The latter substance is prepared by heating stibanilic acid with urea solution, this acid being obtained by the customary alkaline hydrolysis of its acetyl-derivative. It is now found that the published directions for the latter process are insufficiently definite in one important particular, viz., the method of determining when hydrolysis is complete. Products containing varying amounts of unchanged *p*-acetylaminophenylstibinic acid may be obtained according to the conditions chosen for this test, still keeping within the scope of these published directions. When such a product is treated, under suitable conditions, with urea, the stibanilic acid present reacts in two ways:—

(a) With formation of the ureide.

(b) Undergoing hydrolytic fission with formation of antimoniac acid.

The former, in a pure state, does not form appreciably soluble salts; its solubility in presence of the antimoniac and acetylaminophenylstibinic acids is to be attributed to the action of these substances as protective colloids (see § 9). The relative amounts of the three substances in the final product, as well as of urea, which is difficult to remove from these amorphous materials, depend on the experimental conditions. This source of variation accounts for the difficulty in repeating the preparation of "urea stibamine" which has been reported by other investigators (Napier, 1923; Uhlenhuth, Kuhn and Schmidt, 1925), and possibly for the alleged variation in the composition of the samples on the Indian market (Ghosh and others, 1928; Napier, 1929).

4. It will be seen from the foregoing that this problem has been investigated biologically as well as chemically, and the opportunity has been taken to include in the physiological section, on a basis of the results obtained, a general discussion of methods appropriate for the examination of all such chemotherapeutic problems from the physiological standpoint.

5. There is no evidence that the relative values of these products, as estimated by these experiments on mice, are transferable to their use in human kala-azar. For example, we find that stibamine glucoside is less active on trypanosomiasis in the mouse than "urea stibamine," whereas Napier (1929) finds that the two drugs are equal in therapeutic efficacy for kala-azar.

CHEMICAL SECTION.

6. "Urea stibamine (Brahmachari)," was analysed and gave the following results :—

Table I.

Batch No.	Loss on drying.					Atomic ratios.	
		H.	N.	Sb.		C : N.	C : Sb.
425	10.38	—	—	6.75	44.19		
425	10.38	—	—	6.77	44.49		
451	7.14	20.2	3.0				
522	12.17	20.9	2.9				
522	14.07	20.5	2.8				
{ 522	12.17	20.9	3.0	46.4			4.57 : 1
{ 522	16.27	21.53	2.67				—
926	10.12	21.16	2.8	46.8			4.59 : 1
1128	9.45	20.17	2.91	6.47	48.6	3.61 : 1	4.21 : 1

The value found for the ratio C : N shows that the product cannot be ammonium *p*-carbamidophenylstibinate, which requires 2.3 : 1 ; the value of the ratio C : Sb shows that it cannot consist of a pure arylstibinate, but must also contain antimony in inorganic combination. This is shown to be antimonious acid (see § 9). In order that a determination of the ratio C : N should afford trustworthy information as to the nature of the organometallic constituent, the product was treated with dilute hydrochloric acid to remove ammonia or urea. The resulting solid contained C, 20.2 ; H, 2.4 ; N, 3.4 ; Sb, 52.2 per cent., atomic ratio C : N = 6.98 : 1, showing that the trypanocidally active substance is not *p*-carbamidophenylstibinic acid, which requires C : N = 3.5 : 1. Further information was given by a prolonged treatment of "urea stibamine" (4 g.) with dry alcohol, when the portion remaining undissolved (2.95 g.) was found to have the same composition as the solid obtained by the action of dilute acid (C, 20.5 ; H, 3.1 ; N, 3.5 ; Sb, 50.0 per cent., atomic ratio C : N = 6.91 : 1). The difference in the total nitrogen before and after extraction with alcohol consequently represents the ammonia originally present, removed by the alcohol and volatilised during the evaporation. Calculated on the basis of the total stibinic acid present, this corresponds to approximately 0.36 of a molecular proportion. The residue from the extracts (0.51 g.) contained urea (3.7 per cent. by weight of the original material*)

* The statement of Ghosh and others (1928) that "most of the free urea can be washed out in the cold" is therefore not correct.

and a stibinic acid portion of low trypanocidal activity, probably containing acetylaminophenylstibinic acid (see § 10), which was not obtained in quantity sufficient for identification. The undissolved portion was more active than "urea stibamine" itself, and therefore contained the trypanocidally-active constituent.

7. The alternative remaining is that the latter consists of the di-substituted urea, *s*-diphenylcarbamide-4 : 4'-distibinic acid, which requires an atomic ratio of C : N = 6.5 : 1. The formation of this under the conditions of the preparation was seen to be quite feasible, as it had been shown by Young and Clark (1898) that a mono-substituted urea is converted into the corresponding symmetrical di-substituted urea by boiling with water. The correctness of this conclusion was confirmed in two ways :—

- (a) In investigating the action of urea upon *p*-aminophenylstibinic acid, the relative amounts of urea and water, the temperature, and the time of heating, were found to have a marked influence on the composition of the solid product precipitated by alcohol. These factors were not specified by Brahmachari, nor adequately by the other workers who criticised his directions as lacking in experimental detail (Ghosh and others, 1928 ; Niyogi, 1928). When the concentration of the urea was kept low, in order to favour the formation of a di-substituted urea, as in § 8 and § 10, the product had a trypanocidal action equal to or somewhat greater than that of "urea stibamine," whilst a high concentration of urea led to the formation of a comparatively inactive material. Further, the atomic ratios of carbon to nitrogen in these products, after purification by dilute hydrochloric acid, were 6.5 : 1 and 3.4 : 1 respectively, corresponding in the one case to the presence of *s*-diphenylcarbamide-4 : 4'-distibinic acid as organometallic constituent, in the other of *p*-carbamidophenylstibinic acid.
- (b) These two ureides have been synthesised, and the former shown to have the greater trypanocidal activity, although, owing to the fact that it does not form soluble salts and consequently must be administered in suspension, intraperitoneally instead of intravenously, it is not quite so active as might be expected.

8. *Preparation of a Product containing s-Diphenylcarbamide-4 : 4'-distibinic Acid as Trypanocidal Constituent, by means of Urea.*—A paste of freshly-precipitated *p*-aminophenylstibinic acid, containing 13 g. of water, prepared by hydrolysis of the free acid from 10 g. of stibacetin, using 10 per cent.

hydrochloric acid to test the hydrolysing solution, was treated with solid urea (4.2 g. = 3 molecular proportions), the mixture heated at 80° with continuous stirring until a drop no longer gave a precipitate on dilution with water, allowed to stand overnight and treated with alcohol (100 c.c.). The resulting solid required the addition of a little ammonia for solution, and was dissolved in ammonia and reprecipitated by alcohol, after which it was soluble in water. Yield, 5.03 g. (found : C, 16.9 ; H, 2.8 ; N, 3.7 ; Sb, 45.6 per cent.). The trypanocidal action of this was somewhat greater than that of "urea stibamine," and both this and the composition were reproducible (key numbers of batches physiologically tested, G.10 and G.11). Treatment with dilute acid (see § 6) gave a solid containing C, 14.5 ; H, 2.3 ; N, 2.6 per cent., atomic ratio C : N = 6.5 : 1).

9. The low carbon content in this (required, C 28.2) and in the other cases mentioned in this paper, is due to the presence of antimononic acid. This is shown by the fact that the mother liquors contain aniline, which must have been formed by hydrolysis of the carbon-antimony linkage. In a control experiment, it was found that, in the presence of *p*-aminophenylstibinic acid, antimononic acid was reversibly soluble in ammonia. The formation of reversibly soluble protected antimononic acid in this way does not seem to have been described, although both antimononic acid (Jander, 1918) and the arylstibinic acids (Schmidt, 1920) are known to be pronouncedly colloidal. It is somewhat surprising that this fission occurs also to a considerable extent in the cold. A paste of freshly-precipitated *p*-aminophenylstibinic acid containing an equal weight of water was mixed with solid urea (12 molecular proportions). Rapid solution of both solids took place, and treatment with dilute hydrochloric acid gave, not the expected solution of hydrochlorides but a solid, in which the carbon content had fallen from 25 to 19 per cent., with solubility properties simulating those of the "free acid" obtained from "urea stibamine." Here, rearrangement of the urea cannot have occurred (Werner, 1913), so that it is incorrect to assume, as Brahmachari (1924) has done, that insolubility in dilute hydrochloric acid, in this series, indicates the absence of a free amino-group. This was confirmed by the fact that the ratio C : N remained unchanged in this case.

10. *Preparation of a Product Identical with "Urea Stibamine (Brahmachari)."*—The above product shows small differences from the commercial material (a) in the C : N ratio of the organometallic constituent, (b) in solubility in water, (c) in trypanocidal activity, (d) in the amount of antimononic acid present. This difficulty was overcome when it was found that, by suitable

choice of concentration of hydrochloric acid in the test for the point of completion of hydrolysis of the acetylaminophenylstibinic acid, which is not specified in the published methods (Chem. Fabr. von Heyden, 1912; Schmidt, 1922), a certain mixture of aminophenylstibinic acid and acetylaminophenylstibinic acid was obtained which still fulfilled the conditions of this test, and yielded on suitable treatment with urea a product identical with the commercial material in all these respects. The C : N ratio of 7 : 1 is accounted for by the presence of unchanged acetylaminophenylstibinic acid (C : N = 8 : 1); this helps to preserve the solubility of the mixture of weak acids by preventing loss of ammonia on drying. The free acid from 15 g. of stibacetin was hydrolysed, using decinormal hydrochloric acid to test for completion. In a control preparation, the product so obtained was found to contain C, 28.3; H, 3.2; N, 4.7 per cent., atomic ratio C : N = 7.02 : 1. The freshly precipitated product was uniformly suspended in water (916 c.c.) treated with urea (20.6 g. = 10 molecular proportions) and heated at 80° with continuous stirring until a clear solution was obtained (2 hours), allowed to stand overnight, concentrated to 40 c.c. in a vacuum, and treated with dry alcohol (350 c.c.). The solid was dissolved in a little water and precipitated with dry alcohol. Yield, 4.9 g.; readily soluble in water to a very faintly acid solution, resembling that of "urea stibamine (Brahmachari)"; (found: C, 20.7; H, 3.4; N, 4.4; Sb, 48.4 per cent.). Key number G.12. The nitrogen content shows that it contained a little less impurity of urea than the latter, but purification with dilute hydrochloric acid (see § 6) gave a product of the same composition in each case (found: C, 18.3; N, 3.0 per cent., atomic ratio C : N = 7.07 : 1).

11. *The Action of Urea upon p-Acetylaminophenylstibinic Acid.*—A control experiment to ascertain the fate of this acid under the conditions of Brahmachari's preparation, since it is a constituent of the starting material used in § 10, showed that it gradually dissolved, yielding a water-soluble product* which, after the purification with dilute hydrochloric acid described in § 6, contained C, 27.1; H, 3.1; N, 4.3 per cent., atomic ratio C : N = 7.33 : 1. This value for the ratio shows that the soluble material was not pure ammonium *p*-acetylaminophenylstibinate but that a certain amount of de-acetylation

* Brahmachari and Des Gupta (1929) have since stated that urea *p*-acetylaminophenylstibinate is formed when the acid is heated with urea. The properties of the product are not given; the behaviour of *p*-acetylaminophenylstibinic acid, described above, precludes this assumption, which is also seen to be improbable from their method of preparation, which involves heating to 100°.

had occurred. Whether this was followed by the formation of the N-acetyl-derivative of *s*-diphenylcarbamide-4 : 4'-distibinic acid was not determined; the product was, however, of low activity, and therefore not of consequence as a trypanocidal constituent of "urea stibamine."

12. *s*-Diphenylcarbamide-4 : 4'-distibinic Acid.—Stibacetin (9.7 g.) was hydrolysed as in § 8. The alkaline hydrolysed solution was almost neutralised by 2N-hydrochloric acid and treated with half-saturated sodium acetate solution (78 c.c.). A 12.5 per cent. solution of phosgene in toluene (45 c.c.) was then added in three portions, shaking well, and the mixture shaken for several hours. It was then acidified with 20 per cent. hydrochloric acid (50 c.c.) and the solid was washed with 1.2 per cent. hydrochloric acid, yield 6.5 g. It is not appreciably soluble in alkalis or the ordinary organic solvents (found : C, 28.1; H, 2.8; N, 4.8; Sb, 44.6; $C_{18}H_{14}O_7N_2Sb_2$ requires C, 28.2; H, 2.5; N, 5.1; Sb, 44.0 per cent.). Key number G.9. Treatment with cold concentrated hydrochloric acid led to the formation of a substance with the properties of the corresponding stibine tetrachloride, and approximating in composition to this.

13. *Preparation of a Product containing p*-Carbamidophenylstibinic Acid as Trypanocidal Constituent, by means of Urea.—A paste of *p*-aminophenylstibinic acid containing 20 g. of water, from 15 g. of stibacetin, was well mixed with solid urea (45 g. = 22 molecular proportions), and the resulting solution heated for 7 hours at 75°. It was then treated with dry alcohol (270 c.c.). The solid was treated with alcohol until unchanged in weight (6.4 g.) (found : C, 13.9; H, 2.8; N, 4.8; Sb, 52.3 per cent.). The trypanocidal activity of this is considerably less than that of the corresponding preparation of the di-substituted urea, § 8, making allowance for the fact that fission was somewhat more extensive under the conditions of this preparation (key number G.7). Treatment with dilute acid (see § 6) gave in this case a solid containing C, 10.9; H, 1.8; N, 3.7 per cent., atomic ratio C : N = 3.4 : 1.

14. *p*-Carbamidophenylstibinic Acid.*—Freshly-precipitated *p*-aminophenylstibinic acid from 7 g. of stibacetin was dissolved in sodium hydroxide to a bicarbonate-alkaline solution, the sodium salt precipitated by acetone and dried at room temperature until the acetone was just removed. To this

* Whilst this manuscript was in course of preparation two papers appeared : by Niyogi (1928), stating that ammonium *p*-carbamidophenylstibinate and "urea stibamine" are identical, and by Schmidt (1930) stating that they are different in properties, and that Roehl found ammonium *p*-carbamidophenylstibinate to be inactive in kala-azar in the hamster.

was added 4 g. of a fresh sample of potassium cyanate, and water until the volume was 30 c.c. After being clarified it was treated with glacial acetic acid (4 c.c.) allowed to stand 1 hour, diluted with water (40 c.c.), treated with 25 per cent. hydrochloric acid (13 c.c.), filtered, and washed with 1·2 per cent. hydrochloric acid. Yield, 4·3 g. (found : C, 27·1 ; H, 2·8 ; N, 8·7 ; Sb, 42·3 ; $C_7H_9O_4N_2Sb$ requires C, 27·4 ; H, 3·0 ; N, 9·1 ; Sb, 39·7 per cent.). The sodium salt was readily soluble in water (key number G.5). Although insoluble in cold ammonia, when precipitated together with antimonious acid this acid gave a reversibly-soluble product.

15. The possibility was visualised of the occurrence, simultaneously with the process of hydrolytic fission of the carbon-antimony linkage described in § 9, of the addition of the elements of water in the reverse order. Since, in this case, antimonious acid would also be formed, the question was investigated by treatment of the various products, described in this paper, with iodine in presence of excess of sodium bicarbonate in the cold. A negligible absorption took place, no greater than in the case of the stibacetin used as starting material. *p*-Aminophenylstibinic acid, on the contrary, reacted with approximately a molecular proportion of iodine, with formation of *p*-iodoaniline. Although this acid is known to yield *p*-iodoaniline when heated with hydriodic acid (Chem. Fabr. von Heyden, 1912) the formation of this in alkaline solution in the cold is unexpected, in view of the greater stability of the linkage of carbon to antimony under these conditions.

PHYSIOLOGICAL SECTION.

A.—Therapeutic Action.

16. *Methods*.—The therapeutic activity of these compounds was compared in the following way. Mice of 10 to 15 grams weight were infected intraperitoneally with *Trypanosoma equiperdum* (the strain used in this country for standardising the organic arsenicals). About 0·1 c.c. of an approximately 1/50 suspension of the blood of a mouse infected about 3 days before from a guinea-pig carrier was used. The mice were bred in the laboratory and were divided into two groups, so that each mouse in one group had a litter mate in the other group, the number of litter mates in each group being approximately equal. The drugs were injected into the tail vein, one group receiving the drug being examined and the other group receiving, in each experiment, a dose of stibamine glucoside. As a standard of reference in these experiments it was desirable to use a substance belonging to the same class, that is to say, an

organic antimony compound rather than one of the antimonyl tartrates, since the latter have toxic effects of a particular kind which are now supposed to be due to separation of antimony trioxide by the serum alkali. "Neostam" (the nitrogen-glucoside of sodium *p*-aminophenylstibinate) was selected for the purpose, being available in quantity and of constant physiological properties.

17. In the earlier experiments the injection was made at the time when the number of trypanosomes in the blood was about 500,000 per cubic millimetre, but in the course of the investigation the rate of development of infection became so fast that we lost mice by waiting an extra day, and we therefore gave the injection earlier when the infection was just appearing in the blood. No significant difference appears, except in so far as with very heavily infected mice no dose of drug will remove the trypanosomes. Care was taken not to use for infection any strain of trypanosomes which might have developed resistance to antimony by exposure to the action of an antimonial preparation. As with most of the organic arsenic compounds, the action of these drugs on this infection is only temporary, the trypanosomes almost invariably re-appearing in the blood in the course of some days.

18. The degree of infection was judged by microscopic examination of a drop of undiluted blood squeezed between a slide and a coverslip sufficient to cover about one-eighth area of a $\frac{3}{4}$ -inch coverslip, using an 8-mm. apochromat objective and an 18 eye-piece. Arbitrary values expressed by the number of + signs were given to indicate the degree of infection, + representing three to four in twenty fields, ++ representing one trypanosome per field, and 4+ (+++++) the condition when the trypanosomes appeared to be as numerous as the corpuscles and number about 2,000,000 per cubic millimetre. It is essential to use a fairly large number of animals to obtain an estimate of the variation in result between different individuals and it was impossible to count trypanosomes in every animal. Our impression is that the estimate by eye does not introduce an error comparable in dimensions with the variation between different animals. We are convinced that the variability in the comparative curative action of any one of these drugs from one animal to another is only slightly dependent on the degree of infection at the time within fairly wide limits; and, if each test is made in the form of a comparison with a standard, the conditions are similar for both the drugs and the standard of comparison.

19. Some considerations as to factors affecting the accuracy of the method are given in the discussion. There was, however, more tendency in certain of the experiments, where the injection of the drug was made before any trypano-

somes appeared in the blood, for an odd mouse to remain alive without trypanosomes for much longer than any of its fellows in the same group, although, even when the infection was advanced at the time of treatment the same might occur, but more rarely. These odd mice considerably diminish the statistical significance of the difference between groups of mice under comparison, by increasing the calculated standard deviation; and if any way can be found to eliminate them, a considerable increase in the accuracy of comparison is likely. It is possible that the development of some degree of immunity by the mice is responsible for these happenings, and it is not difficult to imagine, from what is known of the precipitate development of immunity to other organisms after a long and variable latent period, that a very slight difference in the conditions for one mouse in a group might make a relatively large difference in its immunity response.

Experimental Therapeutic Results.

20. *Example of Experiments.*—One experiment on the therapeutic comparison will be described with full protocols, but for the rest of the experiments only a shortened account will be given.

Experiment 1.—Two groups of mice, 8 and 10 in number, which were made up of litter mates, were infected with diluted blood from a mouse heavily infected with trypanosomes. The following day one plus (+) was found in the blood of all except five mice, and all were injected. The group of 8 was injected intravenously with “urea stibamine (Brahmachari)” (B.926), the dose being 1.5 mg. per 20 g. of mouse, the weight of the animals varying from 10 to 16 g.; the group of 10 was injected with 3.0 mg. of stibamine glucoside per 20 g. of mouse. The blood was observed each day until all the animals were dead. The history of each mouse in this experiment is given in Table II. The table shows the degree of infection of each mouse, identified by its own number throughout the table, on and after the day of injection of the drug. It will be seen that those animals which showed no trypanosomes on the day on which the drug was injected did not depart in rate of re-infection beyond the range of variation of the other animals.

Table II.—Intensity of Infection in Two Groups of Mice. Mice Nos. 1 to 8 were given 1.5 mg. of urea stibamine B.926; 9 to 18, 3.0 mg. of stibamine glucoside for 20 g. mouse. The first figure for each mouse is the number of plus signs given on the day of injection, the succeeding figures the number of plus signs for each successive day till death (D).

Mouse No. 1	1	0	0	0	0	0	0	0	0	0	0	0	1	2	2	D
" 2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 3 4 D
" 3	1	0	0	0	0	0	0	0	0	0	0	0	0	3	4	D
" 4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3 4 D
" 5	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	3 4 D
" 6	0	0	0	0	0	0	0	0	0	0	0	0	1	3	4	D
" 7	0	0	0	0	0	0	0	0	0	0	0	1	1	2	3	D
" 8	1	0	0	0	0	0	0	0	0	0	0	0	1	2	2	D
9	1	1	0	0	0	0	0	1	3	4	D					
10	1	0	0	0	0	0	1	2	4	4	D					
11	1	0	0	0	0	0	0	0	0	0	0	0	1	3	D	
12	2	0	0	0	0	0	0	0	0	0	0	0	0	0	4	D
13	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1 2 2 3 D	
14	1	0	1	0	0	0	0	1	1	4	4	D				
15	0	0	0	0	0	0	0	1	1	3	4	D				
16	0	0	0	0	0	0	0	0	0	2	4	4	D			
17	1	0	0	0	0	0	0	1	3	D						
18	1	0	0	0	0	0	0	0	0	3	4	D				

21. The difference between the action of the two drugs can be expressed in three different ways, namely, (1) the average time which the mice survived; (2) the average number of days on which no trypanosomes were found in the blood; (3) by a figure which measures, not only those days on which the mice had no trypanosomes in their blood, but also those days on which the trypanosomes were present in varying degrees. This is obtained in the following manner, and we have called it the "total therapeutic effect." For each individual mouse a score is calculated by subtracting the number of plus signs for each day from 5, 5 plus signs being assigned to the day on which the animal was found dead. These figures are added up for each mouse and the average obtained. The means for all three methods are given in Table III, column 4, which shows that in this disease, "urea stibamine," in this dose, gives mean values of (1) survival time, (2) number of days with no trypanosomes, and (3) total therapeutic effect, which are all higher than that produced by 3 mg. of stibamine glucoside. Figs. 1 and 2 give the same information graphically; fig. 1 showing the percentage of animals surviving each day for each drug, and fig. 2 the average number of pluses for the whole group each day.

22. If, however, Table II is referred to again, it will be seen that the survival times overlap in the two groups of animals. Mouse No. 13 of the stibamine

glucoside group, for instance, did not die for 18 days, whereas mouse No. 7 injected with "urea stibamine" died on the fourteenth day. It is clear,

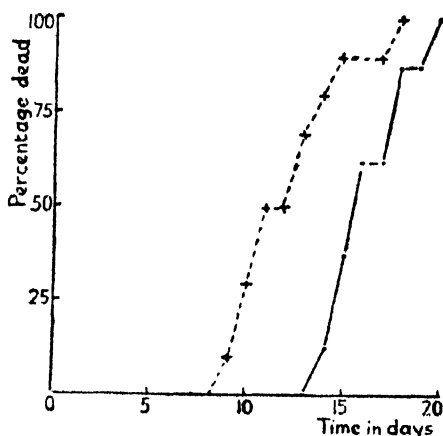


FIG. 1.—Percentage of mice dead on each day after injection of 1.5 mg. urea stibamine (B.926)—(full line) and 3.0 mg. stibamine glucoside W.3 per 20 g. mouse (dotted line).

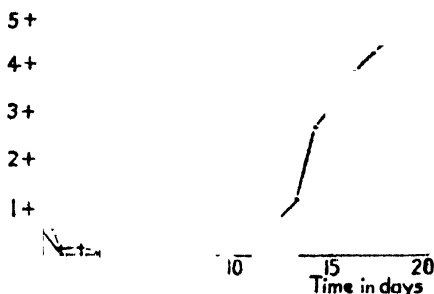


FIG. 2.—Average intensity of infection—number of pluses assigned daily—dead mice being reckoned as 5+. 1.5 mg. B.926 per 20 g. mouse (full line), 3.0 mg. W.3 per 20 g. mouse (broken line).

therefore, that there is a possibility that the difference in *mean* survival time is not due to a real difference in activity of the two drugs, but that the difference has arisen solely by chance amongst the animals used. To determine whether the differences shown in Table III are real differences, or merely random differences due to the variability of mice, we have applied simple statistical tests. The statistical method used is that developed by Fisher from "Students" work (Fisher, p. 109). The ratio of the difference of the average values,

Table III.

Method.	Drug.	Dose.	Mean.	No. of animals.	"t" (see below).	"P" (see below).
Survival time	"Urea stibamine" (B.926)	mg. 1.5	16.5	8	} +3.57	0.0 ₈
	Stibamine glucoside (W.3)	3.0	12.4	10		
Number of days with no try- panosomes	"Urea stibamine" (B.926)	1.5	12.1	8	} +3.24	0.0 ₂
	Stibamine glucoside (W.3.)	3.0	7.9	10		
Average thera- peutic effect	"Urea stibamine" (B.926)	1.5	69	8	} +3.34	0.0 ₁
	Stibamine glucoside (W.3)	3.0	48.7	10		

obtained by either of the above methods, to the standard deviation of the difference, is estimated by the formula

$$t = (m_1 - m_2) \sqrt{\frac{(n_1 + n_2 + 2)(\sum d_1^2 + \sum d_2^2)}{(n_1 + 1)(n_2 + 1)(n_1 + n_2)}}$$

where t is the ratio of the difference of the means to the estimated standard deviation, $(n_1 + 1)$ is the number of animals in one group and $(n_2 + 1)$ the number in the other, $\sum d_1^2$ is the sum of the deviations from the mean of the individual values for one group, and $\sum d_2^2$ the same for the other group. For the experiment described for the mean survival time,

$$t = (16.5 - 12.4)/\sqrt{18 \times 96 \div (8 \times 10 \times 16)} = +3.57$$

the number of animals $(n_1 + 1)$ and $(n_2 + 1)$ being 8 and 10; the sum of the squares of the deviations for the two groups being 96. In this and for all the succeeding experiments the convention has been adopted of subtracting the mean for the standard stibamine glucoside from that of the test group, so that, when the mean for the test group is greater than that for the standard, the sign is positive. The magnitude of the value of " t ," irrespective of the sign, provides a means of judging whether the difference between two such means is statistically significant or not. For large groups of animals the difference is generally taken as being statistically significant if the value of " t " is more than 2.0, values larger than this value only occurring by chance in about 5 per cent. of trials; but for small groups, such as those used, the same degree of significance is only obtained for values of " t " larger than 2. Tables have been published (e.g., Fisher, p. 137) which give the probability "P" of certain values of " t " being exceeded by chance for any given number of animals. The values of " t " and "P" for the three methods of analysis of experiment

are given in Table III. Using Fisher's table, it is found that for this number of animals a value of 3.57 would only be exceeded by chance in less than one in a thousand trials ($P = < 0.001$). This is to say that, unless 1.5 mg. of "urea stibamine" (B.926) is really more active than 3 mg. of stibamine glucoside, such a difference of mean survival times would not arise more than once in a thousand such comparisons as experiment 1; so that there is a high degree of probability that this drug is more than twice as active as stibamine glucoside in mouse trypanosomiasis. Similarly, the differences in the number of days with no trypanosomes would only arise by chance in about the same proportion of trials. The final value obtained is given in the next paragraph. The same treatment is applied to the method of evaluating these results by the number of days with no trypanosomes and by what we have called the "therapeutic effect." The survival time shows a slight advantage in the significance of the results, and this is the general rule (see Table X).

23. The next part of this section (Tables IV, V and VI) gives comparisons, based on survival time, of all the drugs we have tested. In the tables are given first the name of the compound with that of the standard with which it is compared immediately below. The next column gives the identification number of the compound used for cross reference to the chemical section. The third column gives the actual dose of the drug injected for each 20 g. of mouse, the fourth column gives the mean survival time of each group of mice, the fifth column the number of animals injected with each dose, and the sixth and seventh the values of "t" and "P" calculated as previously explained. In the last column is given the amount of the drug equivalent to 1 mg. of the glucoside; and this is either calculated from the ratio of the dose of the drug under test to that of this standard which, in any one comparative experiment, gives a statistically insignificant difference in the survival times of the two groups, by interpolation between two dose-ratios which give significant differences of opposite signs.

24. "*Urea Stibamine (Brahmachari)*."—Table IV, a, b, c, gives the comparisons based on survival time, of three experiments with "urea stibamine (Brahmachari)" (B.926). The drug was compared on one occasion with an equal weight of stibamine glucoside (Table IV, a), on another with twice its weight (Table IV, b, experiment already described), and on a third with three times its weight of stibamine glucoside (Table IV, c). When the ratio of the doses used is unity (Table IV, a) the value of "t" is 6, and such a value could only arise by chance errors of sampling in about one in 10 million such trials. It is certain therefore that this drug is considerably more active than stibamine

Table IV.—Therapeutic Action of various products, as estimated by Time of Survival after Injection of Drugs into Mice Infected with Trypanosomes. Each experiment includes the results on one group of mice treated with the drug to be tested and one group treated with the standard.

Drug.	Key No.	Dose.	Means of survival times.	No. of animals.	"t."	"P."	Dose equivalent to 1 mg. of glucoside.
a. " Urea stibamine (Brahmachari) "	B.926	mg. 1.5	11.8	8	+6	0.01	}
Stibamine glucoside	W.3	1.5	8.4	7			
b. " Urea stibamine (Brahmachari) "	B.926	1.5	16.5	8	+3.57	0.06	}
Stibamine glucoside	W.3	3.0	12.4	10			
c. " Urea stibamine (Brahmachari) "	B.926	1.0	7.26	11	+1.01	0.32	}
Stibamine glucoside	W.3	3.0	6.73	11			
d. " Urea stibamine (Brahmachari) "	B.1128	1.3	9.1	11	+2.13	0.04	}
Stibamine glucoside	W.3	4.0	6.3	9			
e. " Urea stibamine (Brahmachari) "	B.1128	0.75	7.58	12	-3.1	0.002	}
Stibamine glucoside	W.3	3.0	8.6	10			
g. Alcohol-washed " urea stibamine "	B.3	0.75	13.0	11	+0.26	0.86	}
Stibamine glucoside	W.3	3.0	12.8	11			
h. Product containing <i>p</i> -carbamidophenylstibinic acid (urea preparation)	G.7	3.0	20.71	7	1.12	0.28	}
Stibamine glucoside	W.3	6.0	17.57	7			
i. Sodium carbamidophenylstibinate (cyanic acid preparation)	G.5	1.5	9.4	10	0.84	0.42	}
Stibamine glucoside	W.3	3.0	8.75	8			
k. Product identical with " urea stibamine "	G.12	1.0	12	13	+0.13	0.9	}
Stibamine glucoside	W.3	3.0	11.5	11			
l. Product containing <i>s</i> -diphenylcarbamide-4:4'-distibinic acid (urea preparation)	G.10	1.0	9.48	16	+3.34	0.01	}
Stibamine glucoside	W.3	3.0	8.66	12			
m. Product containing <i>s</i> -diphenylcarbamide-4:4'-distibinic acid (urea preparation)	G.10	0.75	5.54	11	+0.52	0.6	}
Stibamine glucoside	W.3	3.0	5.18	11			
n. Product containing <i>s</i> -diphenylcarbamide-4:4'-distibinic acid (urea preparation)	G.11	1.0	11.0	11	0	—	}
Stibamine glucoside	W.3	4.0	11.0	11			
p. <i>s</i> -diphenylcarbamide-4:4'-distibinic acid (phosgene preparation)	G.9	1.4	16.4	9	+1.3	0.21	}
Stibamine glucoside	W.3	3.0	14	7			
q. <i>s</i> -diphenylcarbamide-4:4'-distibinic acid (phosgene preparation)	G.9	1.5	15.6	10	-1.6	0.12	}
Stibamine glucoside	W.3	3.0	23	9			

glucoside in mouse trypanosomiasis. When the ratio of the dose of drug to the dose of stibamine glucoside is reduced to 0.5 the value of "t" is still very high and "P" is proportionately small. When the ratio of the doses injected is 0.33 the value of "t" is 1.01 and the value of "P" is increased to 0.32. Owing to the inevitable variation amongst mice a value of "t" even greater than this might be obtained once out of three times, approximately, from a group of mice divided at random into two sets and treated with the same dose of one drug. We may therefore take it that the therapeutic action of 1 mg. of "urea stibamine (Brahmachari)" in mouse trypanosomiasis is equivalent to that of 3 mg. of stibamine glucoside. Table IV, *d*, *e*, gives the results based on survival times with a second sample of "urea stibamine (Brahmachari)" (B.1128), examined in the same way, and shows that the equivalent of 1 mg. of the glucoside is significantly less than 0.33 and significantly greater than 0.25 mg. We have taken it therefore as the geometric mean (0.29) of these figures. This sample is slightly more active than the previous one.

25. Table IV, *h*, gives the results of the therapeutic test of the product containing *p*-carbamidophenylstibinic acid (urea preparation) (G.7). This preparation has been discussed in the chemical section under § 13. The activity of this preparation in this infection is equal to that of twice the weight of stibamine glucoside.

26. *p*-carbamidophenylstibinic acid, prepared by means of cyanic acid (G.5) (see § 14), was tested in the form of the sodium salt, and the comparison is given in Table IV, *j*. The result shows that its activity is about twice that of stibamine glucoside but only about three-fifths of that of "urea stibamine." These relations have been confirmed in another experiment, reported later. The comparatively low trypanocidal activity of this substance confirms the chemical evidence that it is not the active constituent of "urea stibamine (Brahmachari)"; since the alcohol-washed material (B.3) with C : N = 7 (*loc. cit.*) is slightly more active than the original product (Table IV, *g*). The same substance, prepared by an alternative method (see footnote, p. 59), was found by Roehl to be inactive on kala-azar in the hamster (Schmidt, 1930).

27. As has been explained (§ 7), the active material is probably the di-substituted urea. Two samples of a product containing *s*-diphenylcarbamide-4 : 4'-distibinic acid (urea preparation) (G.10 ; G.11), prepared as described in § 27, were examined. The therapeutic results are given in Table IV, *l*, *m* and *n*, the first two referring to one sample and the last to the

other. 1 mg. of G.10 gives a significantly higher survival time than 3.0 mg. of stibamine glucoside, while the difference between 0.75 mg. of G.10 and 3.0 mg. of stibamine glucoside is not significant. The dose of this product which is equivalent to 1 mg. of stibamine glucoside is 0.25 mg. The sample G.11, which was a duplicate preparation, gives the same ratio.

28. Results with *s*-diphenylcarbamide-4:4'-distibinic acid, prepared by means of the action of phosgene on the amino-acid (§ 12) (G.9), are given in Table IV, *p* and *q*. The compound was not appreciably soluble in alkali; comparison with the soluble products is therefore difficult. It was injected intraperitoneally in suspension in mucilage of acacia. When compared in this way (Table IV, *p*) 0.47 mg. was not significantly different in its action from 1 mg. stibamine glucoside injected intravenously. The value of "*t*" was +1.3. In another experiment (Table IV, *q*) 1.5 mg. of G.9 and 3 mg. of stibamine glucoside were both injected intraperitoneally, the value of "*t*" then being -1.6, suggesting a slightly larger ratio, but the difference is still not significant. The second experiment is shown in fig. 3 as percentage of

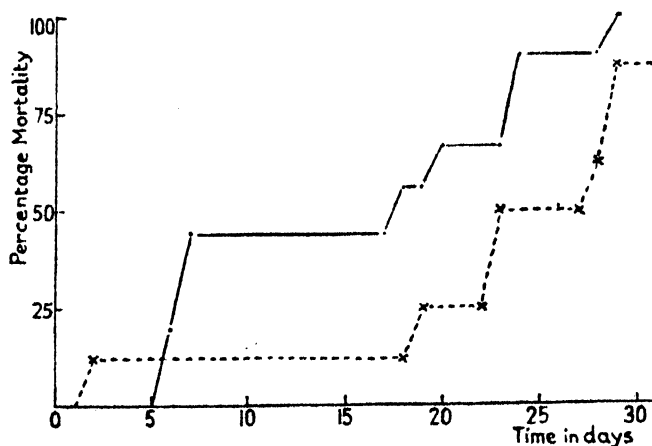


FIG. 3.—Percentage mortality produced in two groups, one injected with 6 mg. G.9 (full line) and the other with 12 mg. of stibamine glucoside W.3.

survivals on each day after injecting the drugs. It will be seen that during the first few days the insoluble drug was less effective than the soluble one, but those that survived the first few days lived practically as long as those injected with stibamine glucoside. The mice were fairly heavily infected before the drugs were administered, and apparently the rate of solution of the insoluble compound was so slow at first, that the infection proceeded to a fatal outcome in some of the animals before a sufficiently high concentration of the drug was

present to inhibit the development of the infection. The dose-equivalent of this substance, with respect to stibamine glucoside, is about 0.5, whereas the soluble preparation containing it, made by means of urea, has a dose-equivalent of 0.25. The fact that one of the two is so sparingly soluble, however, detracts from the value of the comparison.

29. Table IV, *k*, gives the results obtained with the product of the action of urea on partially hydrolysed *p*-acetylaminophenylstibinic acid (G.12) (§ 10). The equivalent dose is the same as that of the commercial "urea stibamine," and the product, as already pointed out, is similar to the commercial material in other ways.

30. Table V gives the results of the comparison of stibanilic acid with stibamine glucoside. Both in the form of the sodium and of the ammonium salts

Table V.

Drug.	Key No.	Dose.	Means of survival times.	No. of animals.	"L."	"P."	Dose. equivalent to 1 mg. of glucoside.
a. Sodium <i>p</i> -aminophenylstibinate (90° preparation)	G.1	mg. 3.3	17	8	+3.87	0.0001	mg.
Stibamine glucoside	W.3	6.0	12.7	11			
b. Sodium <i>p</i> -aminophenylstibinate (90° preparation)	G.1	1.0	8.0	11	-1.6	0.13	0.4
Stibamine glucoside	W.3	3.0	10.9	10			
c. Sodium <i>p</i> -aminophenylstibinate (90° preparation)	G.1	0.75	9.7	13	-3.26	0.0001	
Stibamine glucoside	W.3	3.0	14.1	9			
d. Sodium <i>p</i> -aminophenylstibinate (90° preparation). Kept for 3 months at 37°	G.2	1.5	9.4	11	-0.22	0.85	0.5
Stibamine glucoside	W.3	3.0	9.625	8			
e. Ammonium <i>p</i> -aminophenylstibinate (90° preparation)	G.1	1.2	8.0	10	-0.83	0.42	0.4
Stibamine glucoside	W.3	3.0	8.57	7			

the equivalent dose of the amino-acid is 0.4 mg. A sample of the acid kept in sealed tubes at 37° for 3 months gave a sodium salt with an equivalent dose of 0.5 mg. This is probably significantly different from the original figure, especially as another sample prepared from a different sample of stibacetin showed a similar deterioration.

31. Table VI gives the results of some experiment with stibacetin (W.1), which was the batch used for the preparation of the compounds referred to up to this point. 3.0 mg. is not significantly more active than 4.0 mg. of

Table VI.

Drug.	Key No.	Dose.	Means of survival times.	No. of animals.	"I."	"P."	Dose equivalent to 1 mg. of glucoside.
		mg.					mg.
a. Stibacetin	W.1	3.0	19	9	1.50	0.2	0.7
Stibamine glucoside	W.3	4.0	15	10			
b. Ammonium salt of <i>p</i> -acetylaminophenylstibinic acid	G.14	2.5	13.15	8	+3.0	0.01	} 0.7
Stibamine glucoside	W.3	3.0	8.8	5			
c. Ammonium salt of <i>p</i> -acetylaminophenylstibinic acid	G.14	2.0	10.3	10	-2.2	0.045	
Stibamine glucoside	W.3	4.0	13.0	8			

stibamine glucoside, but since the value of "I" is as much as 1.5, 0.7 mg. is probably nearer the true equivalent of 1 mg. stibamine glucoside than 0.75. The ammonium salt of this preparation has an equivalent dose indistinguishable from this (Table VI, b and c). Another sample of stibacetin (W.2) had somewhat less activity, 0.9 mg. being equivalent to 1 mg. of stibamine glucoside. A series of derivatives prepared from this sample were all less active than those from W.1 (see Table VII). The difference in therapeutic activity persists throughout the series, and although the difference is of an order which is barely significant in each case (see later) yet the fact that it occurs in the same direction for five different pairs of compounds renders it highly probable that the difference is a real one, although there was no corresponding difference

Table VII.

Equivalent dose when made from

	Stibacetin W.1.	Stibacetin W.2.
	mg.	mg.
Ammonium salt of <i>p</i> -acetylaminophenylstibinic acid	0.7	—
Sodium salt of <i>p</i> -acetylaminophenylstibinic acid	—	0.9
Sodium <i>p</i> -aminophenylstibinate	0.4	0.6
Sodium <i>p</i> -aminophenylstibinate. Kept at 37° for 3 months	0.5	0.65
Sodium <i>p</i> -carbamidophenylstibinate (cyanic acid preparation)	0.5	0.6
Product containing <i>p</i> -carbamidophenylstibinic acid (urea preparation)	0.5	1.0

in composition between the members of the pairs. This point seems to us to be of considerable importance, as showing that more remains to be done before the biological control of the preparation of organic antimonials can be dispensed with.

32. *Accuracy of Therapeutic Test.*—The necessity for using a standard on a comparison group in each experiment is shown by many of the experiments, two examples of which are given in Table VIII. The degree of infection in each case was approximately the same, but the experiments compared were done at different times. The first column gives the dose of stibamine glucoside used. 3 mg. gave a survival time of 12·1 days at one time and 7·4 at another. The values of “*t*” and “*P*” worked out in the same manner as before are given, and it will be seen that there is a significant difference. Similarly, 1·5 mg. produced in two different experiments survival times of 5·7 and 8·4 days, which are again significantly different. The difference was not due to differences in degrees of infection in the experiment, for the following reason. An experiment was done later in which the initial infection was less than in the

Table VIII.—Showing Significant Differences between the Effects of the same Dose of Stibamine Glucoside Injected at different times.

Dose.	Mean survival times.		“ <i>t</i> .”	“ <i>P</i> .”
	1st experiment.	2nd experiment.		
mg.				
3	12·1	7·4	2·02	0·05
1·5	5·7	8·4	2·68	0·02

above experiment by a degree which was quite clearly distinguishable, even by the rather crude method of enumeration adopted, and yet the survival time after 1·5 mg. of stibamine glucoside was 8·3 days as compared with 8·4 ; so that lowering the degree of infection does not always produce a difference in survival time. There seemed to be some evidence that the virulence of the infection was increasing during the series of experiments, perhaps on account of rise of room temperature associated with summer weather.

33. On some occasions the group of mice injected divided itself into two, one part of the group dying early with no disappearance of trypanosomes, and the other part losing trypanosomes, if the drug was active enough, and surviving very much longer. Some of these antimony preparations, at any rate, seem

to have a latent period of action, and, if the infection is very great before this latent period is passed, the infection becomes heavy enough to kill the animal before the drug can take effect. -

34. The error of these comparisons can be fairly adequately estimated from an experiment put up especially to determine this point, and from evidence which can be derived from the various repetitions of the experiment on the same drug, already recorded. In the experiment designed to test this point, two groups of 12 and 10 animals were injected respectively with 3 mg. and 4 mg., irrespective of body weight. The results are given in Table IX. Tested by

Table IX.

Drug.	Key No.	Dose.	Means of survival times.	No. of animals.	" t."	" P."	Dose equivalent to 1 gm. of glucoside.
Stibamine glucoside	W.3	mg. 4	20.1	12	+2.6	0.016	—
.....	W.3	3	12.1	10			

all three methods there was a significant difference between these two doses. The failure to adjust to the body weight necessarily increases the standard deviation, because the heavy mice will get too little, and the small mice too much, so that we actually get a lower estimate of the significance of the difference; and it may be taken as fairly certain that doses differing in therapeutic activity in the ratio of 1 to 1.33 can be distinguished, when two groups of 10 animals each are used for comparison, and the results are confirmed by the following evidence.

35. For two drugs the ratio of the dose of the drug to that of the standard was the same in two separate experiments, and the results for both drugs were not significantly different from that of the standard on both the occasions on which they were tried. So far as it goes, therefore, the repetition of the experiment gives the same result, although in both these cases the degree of infection for the two corresponding experiments was quite different.

36. On four occasions the drug was injected in the first experiment in a dose bearing a ratio to the standard of half that which it had in the second experiment. In one the value of "t" for the larger dose was +6, for the smaller dose +3.57; in another "t" was +1.8 for the larger and -2.7 for the smaller dose; for the third "t" was +3.7 and -4.3 for the larger and smaller doses

respectively; in the fourth "*t*" was -0.235 for the larger and -4.6 for the smaller. So that one may expect, on the average, that, if the doses are appropriate, a dose of 2 units will be significantly greater in effect than a given quantity of a standard, and a dose of 1 unit significantly less. That is to say, the ratio which can be distinguished is not greater than 1.414 (the geometric mean of 1 and 2), since the values of "*t*," in general are more than sufficient (2.1 for a value of "*P*" of 0.05) to call the difference significant. This is borne out by further experiments in which the ratios of doses were smaller. In one experiment 1 mg. "urea stibamine" in one case and 0.75 mg. in the other were compared with 3 mg. of stibamine glucoside. In the first case the value of "*t*" was -1.6 , in the second -3.6 . The discrimination is therefore of some ratio less than 1.33. In another set of experiments (experiments 16 and 17), in the first case "urea stibamine" was shown to be significantly more active on trypanosomiasis in the mouse than three times its weight of stibamine glucoside, "*t*" being $+2.13$, and in the second case to be significantly less active than four times its weight, with a "*t*" of -3.1 . The distinguishable ratio indicated in this case is 1.15. That is to say, if all experiments behaved as this one has done, it would be possible to distinguish, on two groups of 10 animals, two similar antimony compounds differing in activity by 15 per cent.; but this estimate is probably unduly favourable. As an approximation, it may be taken that the therapeutic activity assigned as the result of these experiments is on the average not in error by more than 30 per cent., possibly not more than 25 per cent. For the purpose of this paper these limits may be taken as sufficient. To work out accurately the average error would mean a large series of experiments with the same drug, and would only be of interest in connection with the routine standardisation of these and similar compounds intended for therapeutic use.

B.—*Toxicity Tests.*

37. A number of the compounds were examined for toxicity on mice. The drugs were made up in distilled water to such a volume that the required dose was contained in 0.5 c.c. for 20 g. of mouse, the volume being adjusted to the weight of the animal. Distilled water was used because some of the compounds gave cloudy solutions with saline. Comparison of stibamine glucoside in distilled water and saline showed no difference in toxicity. On the average about 150 mice were used in groups of 30 to 50 at different doses for each drug. The doses used were chosen from preliminary experiments so as to fall in the range between doses causing no deaths and those causing the death of all animals injected. In this way a characteristic toxicity curve (Trevan, 1927)

for the drug on mice has been obtained for the more important of the preparations already dealt with.

38. Fig. 4 shows the characteristic curve for stibamine glucoside, for which

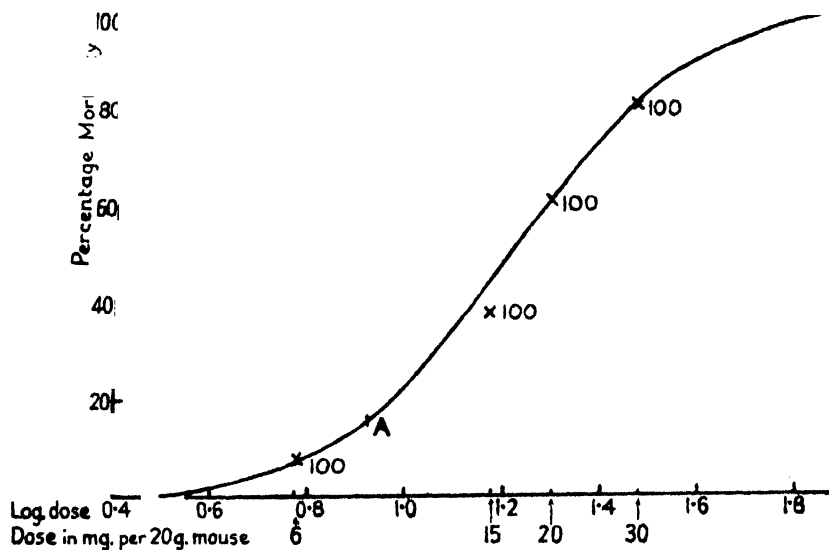


FIG. 4.—Characteristic toxicity curve for stibamine glucoside. Ordinates percentage mortality abscissae logarithm of dose and dose in milligrams per 20 gm. mouse. Numbers at each point represent number of mice used.

400 mice were used. Percentage mortality is plotted against the logarithm of the dose injected. The number of animals injected is indicated by the figure attached to each observed point. The smooth curve is a normal percentile frequency curve; it was fitted in the following manner. A straight regression line was fitted to the observed point in the standard manner. The 50 per cent. mortality ordinate of this line is taken as given the mean lethal dose (the LD 50), and a series of percentile frequency curves of different slopes was then drawn through this point until one was found from which the sum of the squares of the deviations of the observed points was a minimum. The apparatus described by Trotter (1923) for the graphic fitting of curves was found very useful for this purpose. It will be seen that the frequency curve is a very good fit for the observed points. It has been found that if logarithms of doses are plotted against mortality in this way a large number of drugs give a characteristic which is indistinguishable from a normal frequency curve. One of us has previously pointed out (Trevan, 1927) that the shape of these characteristics depends on the distribution of variability of the "individual lethal dose,"

Table X.

Reference to table.	Survival time.	Number of days with no trypanosomes.	Average therapeutic effect.
3b	+3.57	+3.24	+3.34
3c	+1.01	+0.4	+1.07
3d	+2.13	+1.1	+1.55
3e	-3.1	-2.06	-2.5
3g	+0.26	-0.37	—
3h	-0.43	-1.43	-0.705
3j	-2.05	-1.5	-1.9
3k	+0.13	-0.60	-0.27
3l	+1.86	+2.56	+2.3
3m	+0.52	-0.62	+0.51
3n	0	+1.73	+0.52
3p	+1.6	+1.1	—
4a	-3.45	+0.32	—
4b	-1.86	-0.7	-3.16
4c	-1.59	-1.56	-1.83
4d	+0.49	+0.71	+1.0
4e	-2.40	-3.0	-1.8
4g	+3.87	+3.55	+1.46
4h	-1.6	-2.4	-1.97
4j	-3.26	-3.4	-3.65
4k	-0.22	+0.66	—
4l	-0.83	+1.03	—
4t	-0.09	-0.45	-0.74
5b	+0.32	-0.18	0
5c	-2.4	+2.08	—
5e	-4.63	-5.95	-5.2
5f	-3.2	-2.24	-2.8
5g	-1.62	-1.42	—

which is the dose just sufficient to kill each separate member of the groups of mice injected. Thus, for example, in the group injected with a dose of which the logarithm is 0.789 (6 mg.), 8 per cent. were killed. That is to say, of the group of animals injected with this dose 8 per cent. had an "individual lethal dose" of less than 6 mg., and 92 per cent. required more. In the same way, 61 per cent. of animals in the group injected with 20 mg. have an individual lethal dose less than 20 mg., and 39 per cent. greater. Consequently, by graphically differentiating the characteristic curve, close approximations to the distribution of the logarithms of the individual lethal doses would be obtained, and would in this case be the usual bell-shaped normal frequency curve, with the mean at a dose of 16.2 mg. (the logarithm of which is 1.21)—which is the same, for a symmetrical curve, as the median dose producing 50 per cent. mortality (the LD 50).

39. The mean dose is the dose which represents the dose necessary to kill the "average" mouse. What is generally quoted in the literature for organo-metallic compounds is the "maximum tolerated dose," by which is apparently

implied the dose which kills very rarely. In general, no quantitative interpretation of "very rarely" is given, and the attempt is made to determine directly the dose which kills an occasional mouse, when successive groups of 2 to 10 animals are injected with increasing doses. It is obvious that, apart from the general uncertainty arising from the use of small groups of animals, the dose at which the first death occurs will, on the average, be larger the smaller the group of animals used, for the relative frequency with which any dose will give one or more deaths in a group of n animals is $1 - P^n$, where "P" is the probability of survival with this particular dose, and P^n the probability that all will die. Since "P" is always less than 1, $1 - P^n$ is larger the greater the value of " n ," so that for groups of animals injected with increasing doses, the dose which first gives a fatality is likely to be higher when a small group is used, as well to have a larger range on repetition. An estimation of the "maximum tolerated dose," which is not only subject to large chance errors, but depends in a special way on the size of the group injected, seems to us to be fundamentally unsound; and we would propose the following procedure as more logical and having a less ambiguous interpretation. If a variable, such as the logarithm of the individual lethal doses, is distributed in a normal manner, the best estimate of the frequency of the occurrence of any deviation from the average is obtained by calculating its ratio to the standard deviation of the variable, and using the known tables of the frequency of deviation in terms of multiples of the standard deviation. By this means the "maximum tolerated dose" can be defined as the dose which produces an appropriately small percentage mortality, and its estimation will be affected only by an error diminishing with the number of animals used, and distributed normally around the true limiting value of the particular dose chosen. For a normal curve 15.866 per cent. of deviations are less than the mean minus the standard deviation and 15.866 per cent. greater than the mean plus the standard deviation. So that the standard deviation of the logarithms of individual lethal doses can be determined graphically from the characteristic by finding the logarithm of the dose which gives 15.866 per cent. mortality (A, fig. 4) and subtracting it from the logarithm of the dose causing 50 per cent. mortality. For stibamine glucoside the logarithm of the dose causing 15.86 per cent. mortality is 0.92, point S on curve, and the standard deviation is $(1.21 - 0.92) = 0.29$.

The next point to decide is, what multiple of the standard deviation is to be subtracted from the mean to arrive at a reasonable criterion of the "maximum tolerated dose." The choice must be more or less arbitrary, but, once

arrived at, it will render the comparison between the results of different workers of more value. We suggest that the maximum tolerated dose should be taken as the dose which kills approximately 1 in 742 of mice injected. This odd figure is chosen because it corresponds to an integral multiple of the standard deviation, and the dose giving this mortality is obtained by subtracting three times the standard deviation of the logarithms of the individual lethal dose from the logarithm of the median dose. We would suggest that the abbreviated LDO be used for this dose. It is of course, to be clearly distinguished from the Lo used in the titration of diphtheria antitoxin. For stibamine glucoside three times the standard deviation is 0.87 and the logarithm of the maximum tolerated dose as defined above is $1.21 - 0.87 = 0.34$; the maximum tolerated dose is therefore 2.19 mg.; one in 740 mice will be killed by a dose about 2.19 mg. The estimate suffers from an uncertainty, due to the ordinary random errors of sampling of mice, but is, we think, preferable to the usual method, which suffers not only from these errors but from random definition also.

40. Table XI gives the median lethal dose (column 2), the maximum tolerated dose LDO as defined above (column 3) and the standard deviation of the logarithm of the dose (column 4). The average toxicity as judged by the median lethal dose is not widely different except for stibamine glucoside which is less than half as toxic as the next (stibacetin) and is about a third of the toxicity of the aminophenylstibinic acid. The masking of the amino-group by glucose in stibamine glucoside is presumably the cause of this low toxicity.

Table XI.—Toxicity for Mice.

	No. of animals.	Median lethal dose, mg. per 20 g. mouse.	LDO, mg. per 20 g. mouse.	Stand- ard devi- ation log. dose.	Com- parative thera- peutic value.
Stibacetin (W.2)	165	7.69	2.02	0.167	2.70
Stibacetin (W.1)	115	6.49	1.93	0.175	2.76
Sodium <i>p</i> -aminophenylstibinate (G.1)	150	4.95	1.38	0.185	3.45
Sodium <i>p</i> -aminophenylstibinate (G.3)	131	5.37	0.478	0.354	0.96
"Urea stibamine" (B.1128)	167	6.31	1.20	0.24	4.13
Product identical with "urea stibamine" (G.12)	84	6.76	2.24	0.16	6.79
Product containing <i>s</i> -diphenylcarbamide 4 : 4'- distibinic acid (G.10)	127	4.27	0.933	0.22	3.73
Product containing <i>s</i> -diphenylcarbamide 4 : 4'- distibinic acid (G.11)	157	3.98	0.872	0.22	3.49
Stibamine glucoside (W.3)	400	16.2	2.19	0.29	2.19
Diethylamine antimonate (G.15)	224	5.37	1.55	0.18	—

A similar but less marked diminution in toxicity is brought about by the insertion of an acetyl-group in *p*-aminophenylstibinic acid (compare G.1 and stibacetin W.1 from which this was prepared). The difference between these is probably statistically significant. A similar difference is to be observed between the LD 50 of stibacetin W.2 and that of *p*-aminophenylstibinic acid G.3 which was prepared from it.

41. The product containing *s*-diphenylcarbamide-4 : 4'-distibinic acid (urea preparation) (G.10, G.11), on the other hand, has as high a toxicity as the amino-acid and the therapeutic activity is still more enhanced. The corresponding product made from partially hydrolysed acetyl-derivative (G.12) as well as "urea stibamine (Brahmachari)" on the other hand, are both of the same order of toxicity as stibacetin. This could be accounted for by assuming that G.12 contains a substance with a lower toxicity than that of stibacetin, resulting from the action of urea on *p*-acetylaminophenylstibinic acid. As stated in § 16, however, this seems very improbable, and we cannot at present suggest a cause, unless the difference in toxicity is due to a difference in physical condition, an explanation we advance with some reluctance.

42. The figures for the standard deviation of the logarithm of the dose show some curious anomalies. The standard deviation varies from 0.18 (diethylamine antimonate) to 0.354 (*p*-aminophenylstibinic acid, G.3) for the different drugs. This difference is certainly a significant one. The dose just sufficient to kill individual mice therefore varies very much more for *p*-aminophenylstibinic acid than for diethylamine antimonate. The dose of the former acid just sufficient to kill about 1 in 740 mice (the LDO, see Table XI) is 0.478 mg., of the latter, 1.55. The dose, on the other hand, which will *fail* to kill only 1 in 740, which we will call the LDC, and which can be estimated by a method analogous to that used for LDO, is 18.6 for diethylamine antimonate and 62 for amino-acid already referred to (G.3). The last figure seems at first sight ludicrous, but examination of fig. 5 shows that it is the inevitable deduction from the toxicity curve as determined, and a similar but less extensive spread is shown by stibamine glucoside (2.19 to 120 mg.), which is based on a much larger number of animals. The LDO of the amino-acid is therefore considerably lower than that of diethylamine antimonate, whereas the LDC is very much higher. The standard deviations of the logarithm of the dose can be divided roughly into three classes (1) those about 0.16 to 0.18, namely, stibacetin, one of the amino-acid preparation, diethylamine antimonate, and the product G.12 (see tables); (2) those from 0.22 to 0.24, the products of G.10, G.11 and B.1128; and (3) stibamine glucoside (0.29). The cause of

this difference in the variability of the response of the mice is obscure. In the last column we have given a comparative "therapeutic index" based on the

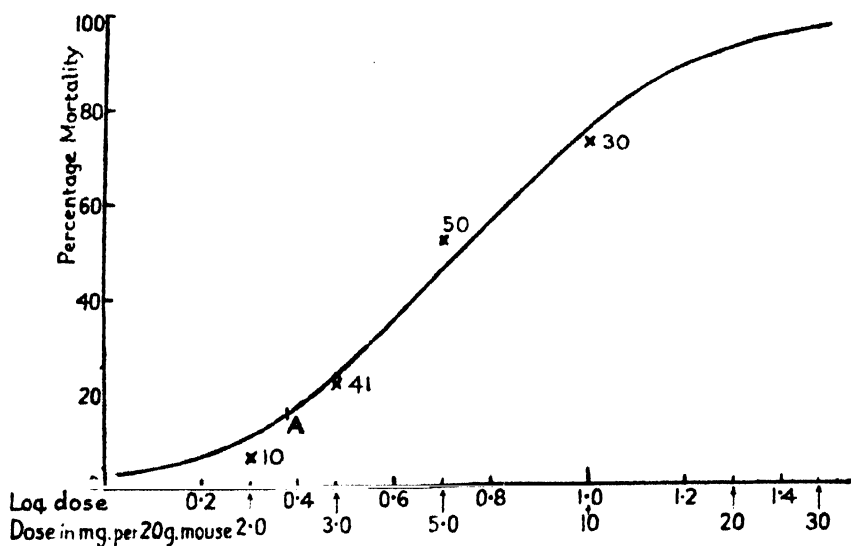


FIG. 5.—Characteristic toxicity curve for amino-acid G.3.

therapeutic effect of 1 mgm. of stibamine glucoside as the standard. The therapeutic index is calculated by dividing the LDO by the dose of the particular drug which is equivalent to 1 mg. of stibamine glucoside in therapeutic effect. The relative value obtained for the ratio between toxicity and therapeutic effect of two drugs must depend on the particular dose selected as representing the maximum tolerated, when the toxicity curves for the two drugs differ in slope. For example, we will take the two stibanilic acids G.1 and G.3. If we had chosen the LD 50 (median lethal dose) as the maximum tolerated dose, the therapeutic value would be $4.95/0.4 = 12.75$ for G.1 and $5.37/0.5 = 10.74$ for G.3—practically equal instead of in the ratio of 3.5 to 1; and if we had felt that a chance of one mouse in 730 dying was too high to call the dose producing that result the maximum tolerated dose (although it is probably a more stringent requirement than is usual), and decided that four times the standard deviation should be used for the calculation of the maximum tolerated dose, the advantage of G.1 over G.3 would have been increased. Again, the increase of the therapeutic index of the urea preparation G.12 over "urea stibamine" B.1128 (Table X) is almost entirely due to the smaller variability of response of mice to the former drug.

Summary.

Urea, in aqueous solution, is found to act upon *p*-aminophenylstibinic acid in two ways :—

- (1) With hydrolysis at the bond between antimony and carbon and the formation of antimoninic acid. This can occur in the cold, as well as above the temperature of rearrangement of urea.
- (2) With formation of the mono-substituted urea, *p*-carbamidophenylstibinic acid, or the di-substituted urea, *s*-diphenylcarbamide-4 : 4'-distibinic acid, according to the conditions of the experiment and the quantities of the reacting substances chosen. This occurs between 75° and 100° C.

These two ureides have also been prepared by the action of cyanic acid and phosgene, respectively, upon *p*-aminophenylstibinic acid, and the second has been shown, by comparison of the therapeutic action of all these products upon a *Trypanosoma equiperdum* infection in mice, to be the protozoicidal constituent of the drug known as "urea stibamine," which was stated by Brahmachari to consist of the ammonium salt of *p*-carbamidophenylstibinic acid ; the latter is found to be considerably less active, so that it is important to fix the conditions of the reaction with urea, so as to obtain the di-substituted urea. As available in commerce, "urea stibamine" is found to contain, in addition to the di-substituted urea, antimoninic acid, as mentioned above, together with some *p*-acetylaminophenylstibinic acid, resulting from incomplete hydrolysis of the stibacetin used as the starting substance ; owing to the weakly acid character of these materials, they retain only a small amount of ammonia, which suffices, in consequence of their predominantly colloidal nature, to bring the whole into solution.

The accuracy of comparison of therapeutic activity on trypanosomiasis in the mouse is discussed. It is shown that the comparison of survival times after treatment with these drugs is at least equal in value to any other method. The toxicity of a number of the products has been determined, using for this purpose a larger number of animals than has usually been employed, and characteristic toxicity curves obtained. The "maximum tolerated dose" is rigidly defined, and is chosen as the dose which kills 1 in 742 of the mice. Comparative therapeutic values are obtained by dividing the maximum tolerated dose of each drug, as so defined, by the dose which is equivalent in trypanocidal action to the standard dose of the reference substance, stibamine glucoside.

The authors desire to express their warmest thanks to Dr. T. A. Henry for his kind advice and criticism, to Mr. A. Davies for help in preparation and analysis of the compounds described, and to Miss Westbrook for help with some of the animal experiments.

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*On the Optimum Hydrogen Ion Concentration and Temperature of
the Style Enzyme of Pecten maximus.*

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Within the past decade there has been a considerable increase in the amount of work produced dealing with the processes of digestion in many groups of animals and more especially of invertebrates. Despite the continuous increase in the interest taken in the subject, and in the number of workers engaged in research on this aspect of invertebrate physiology, there has been little corresponding advance in the methods of investigation employed, and few general results have been obtained. Facts concerning enzymes known to biochemists have only too frequently not been appreciated by biologists. The most frequent method of investigating the digestion processes of an animal has been the preparation of a crude extract of an entire gut or gland by grinding with sand and diluting either with distilled or with sea water, setting up a series of such preparations—which possess a hydrogen ion concentration of unknown meaning—at temperatures varying from 0° C. to 100° C. for a length of time dictated more by convenience than by significance, and determining, frequently by not the most delicate method, the amount of digestion that has occurred. The investigation of effects due to the hydrogen ion concentration has been mostly conducted at that temperature which had been previously found to give maximal digestion and for a length of time again devoid of meaning. As a result of this method of procedure, it is often hard to correlate the optimal p_H values and temperatures obtained with those that are actually to be encountered in the living animal.

In the digestion of foodstuffs by enzyme action there are in all five variable factors upon which the process depends :—

- (1) The strength of the enzyme in the digestion mass.
- (2) The concentration of the substrate.
- (3) The temperature at which digestion is carried on.
- (4) The hydrogen ion concentration of the medium.
- (5) The time for which the enzyme acts.

The interdependence of several of these factors has been shown by numerous workers. It has long been known that the activity of an enzyme depends on the temperature at which it is working and also on the hydrogen ion concentration of the medium. Sørensen (1909) using the invertase obtained from vertebrate liver showed that the optimum hydrogen ion concentration varied with the length of time for which the enzyme was allowed to act, and O'Sullivan and Thompson (1890) also using invertase proved that it was dependent on the temperature at which it acted. A reciprocal connection between hydrogen ion concentration and temperature was later discovered by Compton (1915) working with taka-diastrase. The relationship between optimum temperature and time has been recently worked out by Berrill (1929) for the amylolytic enzymes of tunicates, demonstrating that the longer the enzyme is allowed to act the lower becomes its optimum temperature. Miss Nicol (1930) has repeated some of the work of both Compton and Berrill with the diastase of *Sabella pavonina*, but obtained no definite positive results.

Apart from the work of Berrill and of Miss Nicol just referred to, very little endeavour has been made by zoologists to relate the various factors under which animal enzymes work. By far the greater number of p_H and of temperature optima that have been published recently, therefore, are special cases—statements that at a definite p_H and for a certain digestion time a certain temperature is optimal for the activity of the enzyme, and only too frequently the cases selected have little biological significance. The optimum hydrogen ion concentration for an enzyme being dependent on the temperature at which it is working, it is a point of purely academic interest that a certain p_H is optimal at a temperature which the animal can never experience. In order that the results given in digestion work may be significant, the experiments must be based on preliminary investigations determining the time for which the food is exposed to the action of the enzyme in the alimentary canal of the animal, the actual hydrogen ion concentration of the gut, and the temperature at which the animal normally lives. The other two variables—concentration of the substrate and of the enzyme—are beyond the control of the animal and are therefore of only secondary interest to biologists.

An attempt has been made, in the series of experiments about to be described, to investigate more fully the conditions that determine the optimum p_H and temperature values, and to correlate these with the actual conditions found in the living animal. For this purpose the enzyme chosen was the diastase contained in the crystalline style of the scallop, *Pecten maximus*. This enzyme was selected because it was easy to obtain in large quantities, was of

considerable strength, and was by far the purest invertebrate enzyme obtainable. In addition, it was a diastase the estimation of the activity of which meant the estimation of reducing sugars, for which more dependable methods exist than for the estimation of other digestion products. *Pecten maximus* was chosen as the source of the style as it could be easily obtained at Plymouth and because the style was a more or less permanent structure. The animals were kept in circulating water and remained healthy; 3 or 4 days were allowed to elapse after the molluscs were brought in by the trawler before any of that batch were used for experimental purposes, as it was found that that time was required for the elaboration of a new style, the old one being lost on transference from the sea to aquarium conditions.

The styles were removed from the desired number of individuals by cutting into the stomach through the digestive diverticula and picking out with a pair of forceps. They were carefully washed in sea water to remove any adherent tissue of the digestive diverticula, the mid-gut or the gonad, which might have introduced other enzymes. The styles were then dried on a clean piece of butter muslin, weighed, ground up with a small quantity of silver sand that had been freshly washed either with distilled or with sea water, and made into an extract of usually 0.3 to 0.5 per cent. by weight, by addition of either distilled or sea water, according to circumstances.

The temperatures of the experiments were controlled by the use of a thermostat that was found to be constant to about 1° C. over a 6-hour period. Measurements of the temperature were taken at the beginning and at the end of each experiment, the mean of the two values being used when plotting curves. For temperatures lower than 20° C. use was made of the circulation of sea water at Plymouth which was found to be constant to 0.1° C. over long periods, of an ice chest and of ice and water mixtures.

The hydrogen ion concentration of the medium was controlled at first by making a sea water extract of the styles and adding HCl or NaOH directly, the p_H being measured colorimetrically by Clark and Lubs' indicators. It was found that such systems were not sufficiently buffered and that incubation at temperatures over 30° C. led to considerable rise in the p_H , presumably due to carbon dioxide being driven off by heat. This occurred even after the mixture had been thoroughly equilibrated by bubbling compressed air through it for 30 minutes, and also after letting enzyme and substrate stand for an hour at 30° C. before mixing. Recourse was therefore had to buffer solutions; those of Walpole (Clark, 1924), of Palitzsch (Clark, 1924), of Atkins and Pantin (1926), and of Northrop as modified by Miss Nicol (1930) were used, the first

and the last proving the most useful, borax or borate buffers seemingly interfering with the digestion processes. By these means the p_H of the experimental tubes was kept steady throughout the course of the experiments, colorimetric measurements of 2 c.c. lots at the beginning and at the end of the experiments giving almost identical tints.

As substrate 1 per cent. starch solution free from reducing sugar was used, and the experiments, which were carried out in test-tubes of 15 c.c. capacity closed with clean corks, were sterilised by the liberal addition of toluol. The sugar resulting from the digestion of the starch was estimated by the modification of the Hagedorn and Jensen (1923) method devised by Boyland (1928). This is a much more satisfactory method of measuring reducing sugars quantitatively than that of Benedict, which has been so extensively used, as the final titration is an iodine-thiosulphate one, giving a sharp end-point and so allowing accurate results to be obtained.

In the experiments the relations between the concentration of the enzyme and of the substrate and the other variables were not investigated, as it is improbable that they are under the control of the animal. The amount of food eaten varies quite without reference to any other of the factors, while the secretion of enzyme is also probably variable without significance.

All experiments were rigorously controlled.

(a) Relation between Duration of the Experiment and Optimum Temperature.

This relationship was not investigated. It has been known since Blackman published his results in 1905 that the optimum temperature shows a gradual lowering with increase in the duration of the experiment.

(b) Relation between Duration of the Experiment and Optimum p_H .

Sørensen (1909) described a change in the optimum hydrogen ion concentration for the activity of the invertase from a vertebrate liver. This change occurred between 2 and 3 minutes after the commencement of the experiment; it can therefore be regarded as of only chemical interest, and it does not seem possible that such a change in optimum p_H could have any importance from the biological point of view.

In order to investigate the possibility of change in the optimum p_H with variation in the time over which the experiment was conducted, the following series of experiments was performed:—

Controls.—5 c.c. buffer + 5 c.c. 1 per cent. starch + 1 c.c. 0.3 per cent. style extract + toluol, boiled and left 51 hours at 30° C. Estimation of sugar in 2 c.c. lots.

Experiments.—5 c.c. buffer + 5 c.c. 1 per cent. starch + 1 c.c. 0.3 per cent. style extract + toluol, left at 30° C. for 8, 16, 24, 32, and 51 hour periods, boiled. Estimation of sugar in 2 c.c. lots.

These results when plotted so as to show the relationship between p_H and the amount of starch digested give fig. 1, and they show very plainly that p_H 6.5

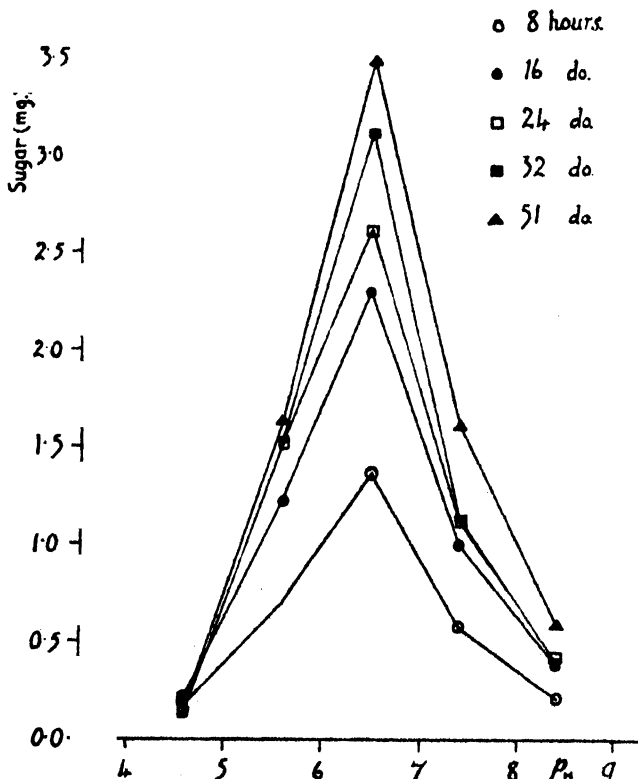


FIG. 1.—Curve illustrating the relation between optimum p_H and the duration of the experiment. Each curve represents a time value. ○, 8 hours; ●, 16 hours; □, 24 hours; ■, 32 hours; ▲, 51 hours.

is optimal by a considerable excess over the others for all times from 8 to 51 hours. When plotted so as to bring out the relation between time and the amount of starch digested they give fig. 2. Here, throughout the course of the experiment, the curve for p_H 6.5 is higher than the others. The curve for p_H 5.6 is also throughout higher than that for p_H 7.4, showing that digestion

is generally more rapid on the acid side than on the alkaline. As there is no intersection of the curves, there is no change in the optimum p_H , with variation in the time of exposure of the substrate to the enzyme.

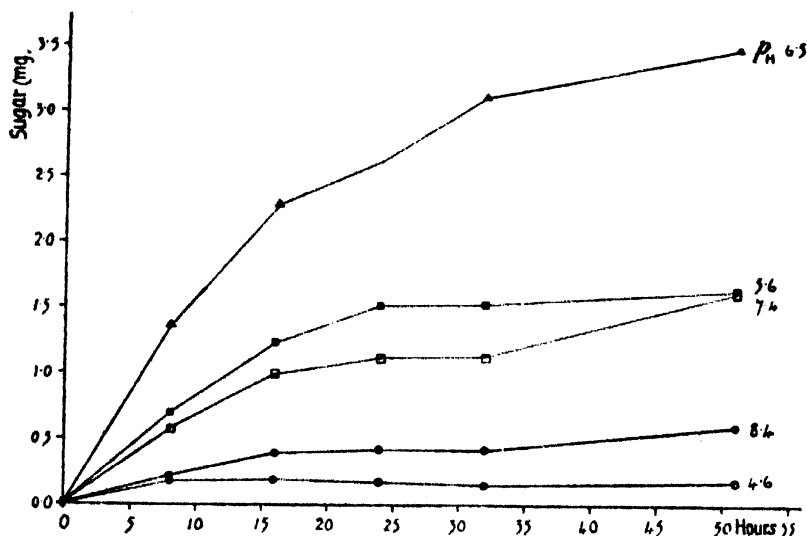


FIG. 2.—Curve illustrating the relation between optimum p_H and the duration of the experiment. Each curve represents a p_H value. ○, p_H 4.6; ■, p_H 5.6; ▲, p_H 6.5; □, p_H 7.4; ●, p_H 8.4.

(c) Relation between p_H and Optimum Temperature.

In order to investigate this relation, the following series of experiments was performed. In it the strength of the enzyme solution and the duration of the experiment are admittedly merely convenient, as they were found to give a reasonable amount of reducing sugar to estimate, using 2 c.c. as an aliquot part, and as the thermostat was very constant over this period.

Controls.—5 c.c. buffer + 3 c.c. 1 per cent. starch + 2 c.c. 0.3 per cent. style extract + toluol, boiled, left at temperatures as in the following figures for 6 hours. Sugar estimated in 2 c.c. lots.

Experiments.—5 c.c. buffer + 3 c.c. 1 per cent. starch + 2 c.c. 0.3 per cent. style extract + toluol, left at temperatures as in the following figures for 6 hours, boiled. Sugar estimated in 2 c.c. lots.

Experiment 1 buffered at p_H 7.3, when plotted so as to show the relation between temperature and starch digested, gives fig. 3 with an optimum temperature at 38° C.

Experiment 2, buffered at p_H 6.2, when similarly plotted, gives fig. 4 with an optimum temperature at 36° C.

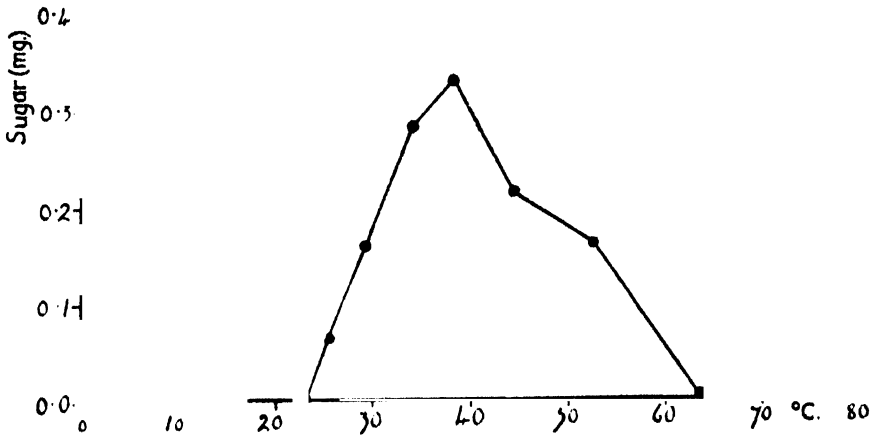


FIG. 3.—Curve illustrating the optimum temperature at p_H 7.3.

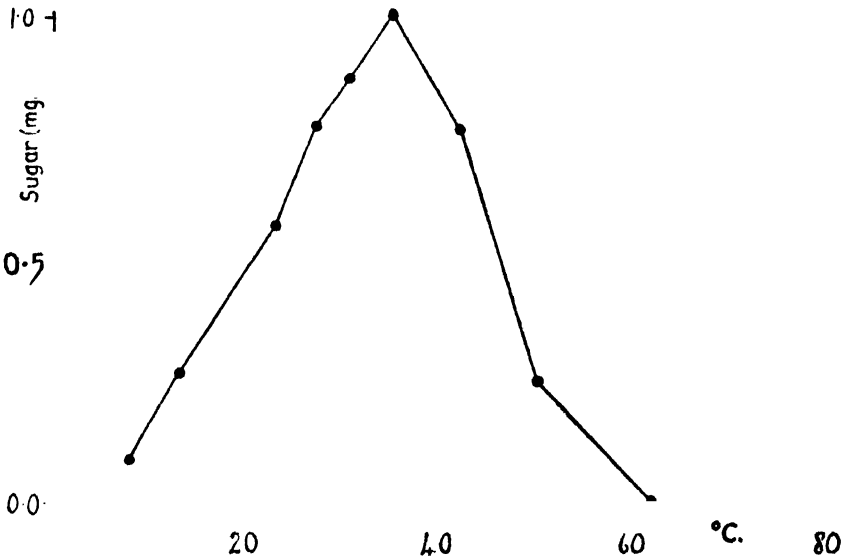


FIG. 4.—Curve illustrating the optimum temperature at p_H 6.2.

Experiment 3, buffered at p_H 4.8, gives an optimum temperature at 26° C., fig. 5.

These three experiments were all conducted separately with enzyme preparations from different batches of molluscs. In order to standardise conditions further the following experiment was carried through, using one preparation

of enzyme throughout, so that the ratio of enzyme to substrate was identical in each experimental tube.

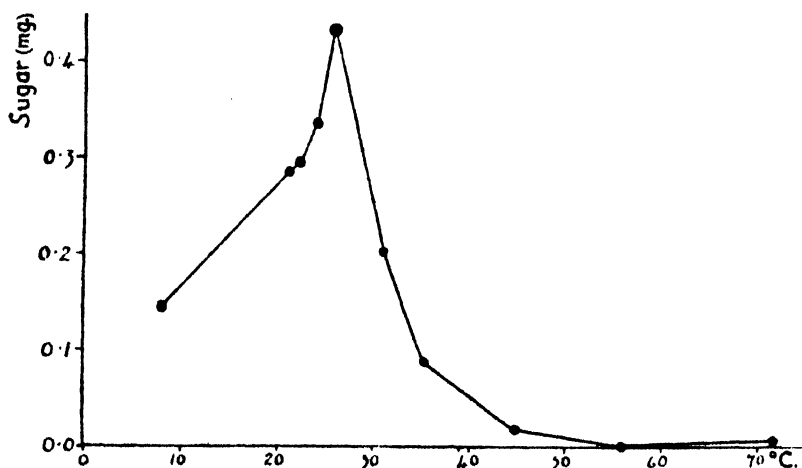


FIG. 5.—Curve illustrating the optimum temperature at p_H 4.8.

Controls.—5 c.c. buffer + 3 c.c. starch 1 per cent. + 2.5 c.c. 0.3 per cent. style extract + toluol, boiled, left at temperatures as in fig. 6 for 6 hours. Sugar estimated in 2 c.c. lots.

Experiments.—5 c.c. buffer + 3 c.c. 1 per cent. starch + 2.5 c.c. 0.3 per cent. style extract + toluol, left at temperatures as in fig. 6 for 6 hours, boiled. Sugar estimated in 2 c.c. lots.

The results of three experiments, buffered at p_H 4.4, p_H 5.3 and p_H 6.8, when plotted to show the relationship between temperature and amount of starch digested, give the three curves in fig. 6. Though in two of them, those for p_H 5.3 and p_H 6.8, the absolute optimum has obviously been missed, yet there is a clear indication of a gradual rise in the optimum temperature for the activity of the enzyme with a rise in the p_H of the medium. The more alkaline the medium, the higher the optimum temperature.

The following point must be carefully noted. When all the experimental tubes are buffered at p_H 4.4, then, *among these*, that incubated at about 25° C. will show maximal digestion; similarly, when all are buffered at p_H 6.8, then, *among these*, that incubated at about 35° C. will show maximal digestion. But that tube, buffered at p_H 6.8 and incubated at 25° C., though not optimal among its own lot, will show more digestion than even the optimal one from those buffered at p_H 4.4. The 25° C. optimum is only an optimum in relation to other tubes at the same or similar hydrogen ion concentrations.

There is therefore a direct relationship between the p_H of the experimental medium and the temperature optimum recorded, and, as is emphasised by

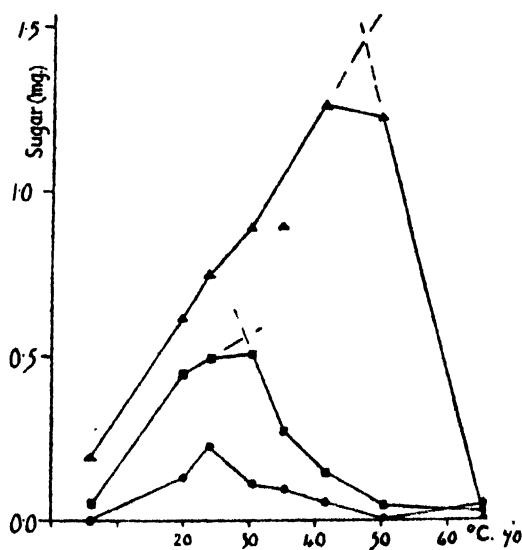


FIG. 6.—Curve illustrating the relation between optimum temperature and the p_H of the experiment. Each curve represents a p_H value. ●, p_H 4.4; ■, p_H 5.3; ▲, p_H 6.8.

Compton (1915), the hydrogen ion concentration of the medium must be specified when any figure is given as the optimum temperature for an enzyme.

(d) *Relation between Hydrogen Ion Concentration, Temperature and Duration of the Experiment.*

In order to correlate the three variables—time, temperature and hydrogen ion concentration—with the conditions actually found in *Pecten*, the following type of experiment was performed. Preliminary investigations had shown that the mollusc lived normally at a temperature more or less constantly of 13° C., and that the food in the gut was in probable contact with the style enzyme for a period of about 10 hours. A series of experiments was therefore carried out, in which the enzyme was allowed to act for the length of time over which it acts in the scallop and at a temperature as close to that at which the animal normally lives as it was possible to obtain. Under such circumstances the p_H optimum was to be determined.

Controls.—5 c.c. buffer + 4 c.c. 1 per cent. starch + 2 c.c. 0.5 per cent. style extract + toluol, boiled, left at 15.2° C. for 10 hours. Sugar estimated in 2 c.c. lots.

Experiments.—5 c.c. buffer + 4 c.c. 1 per cent. starch + 2 c.c. 0.5 per cent. style extract + toluol, left at 15.2° C. for 10 hours, boiled. Sugar estimated in 2 c.c. lots.

The results are shown in fig. 7. The optimum hydrogen ion concentration under such conditions is in the neighbourhood of p_H 5.8. Taking the figures given by Yonge (1925)—and verified for this batch of animals—the hydrogen ion concentration of the stomach contents of *Pecten maximus* and of the mid-gut, the two regions of the Lamellibranch gut where the style enzyme is effective,* are respectively p_H 5.6 and p_H 6.0.

Under the correct natural conditions of time and of temperature, the optimum hydrogen ion concentration experimentally found for the style enzyme is discovered to be the actual concentration encountered in those parts of the alimentary canal of the animal where that enzyme is normally effective.

In the case of extra-cellular enzymes, the optimum p_H as usually determined is never very remote from the p_H of that portion of the alimentary tract where the enzyme is found—in *Pecten*, for example, the optimum as determined at a temperature of 35° C. for a period of 6 hours is about 6.8, a little more than one unit of p_H from that of the stomach. In the case of intracellular enzymes, however, the optimal hydrogen ion concentration and temperature are often values at which the animal could certainly not exist in a healthy state. Thus Yonge (1926) for the protease of the digestive diverticula of the oyster gives optimum p_H values of 3.7 and 10.8. Yonge (1924) has also given the optimum temperature for the enzyme of the hepatopancreas of *Nephrops* as 53° C. While it is admitted that the conditions surrounding an intracellular enzyme, once the tissue containing it has been destroyed as a tissue, present few, if any, points of comparison with those

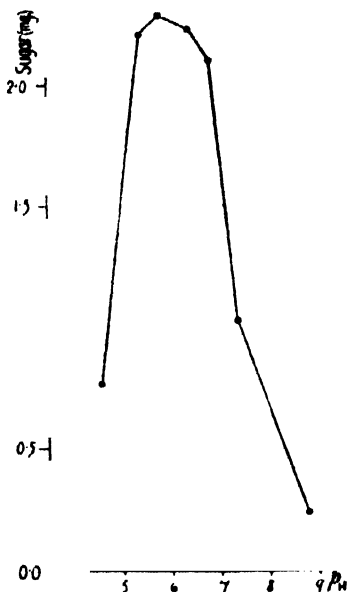


FIG. 7. — Curve illustrating the optimum p_H at natural temperature and duration.

* Absorption of the products of digestion, as Dr. Yonge has pointed out, is probably confined to the digestive diverticula.

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in the intact cell, there is a possibility that if one could determine the true time for which the enzyme normally acts on the ingested particles, the temperature at which the animal normally lives and the actual hydrogen ion concentration of the interior of the cell, then the optimum p_H and temperature for the enzyme would be found to correlate with the natural conditions in these cases as in the case of *Pecten*. It is, in any event, hoped to investigate the case of such an enzyme more thoroughly.

Summary.

(1) The diastase of the crystalline style of *Pecten maximus* has been studied to investigate :—

- (a) The effect of time on the p_H optimum.
- (b) The effect of p_H on the temperature optimum.
- (c) The effect of time and temperature on the p_H optimum.

(2) The principal results are as follow :—

- (a) There is no variation in the p_H optimum with variation in the time of the experiment.
- (b) There is a fall in the optimum temperature accompanying a fall in the p_H of the medium.
- (c) When two out of the three factors—time, p_H , and temperature under the control of the animal—namely, time and temperature, are made equal to those in natural conditions, and the optimum of the third is determined, it is found to be the actual condition encountered in the living animal.

The work described in the previous pages was carried out at the Plymouth Laboratory. My thanks are due to the British Association for permission to use their table there ; to Dr. E. J. Allen, F.R.S., and to Dr. C. M. Yonge for help and encouragement while working there, and in particular to Mr. A. D. Hobson for continued suggestions and advice.

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The Physiological Action of Cyanide.—I. The Effects of Cyanide on the Respiration and Sugar Content of the Potato at 15° C.

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I. INTRODUCTION.

The interest which attaches to the physiological action of cyanide arises from the strong inhibitory effect upon the respiratory processes of the cell. This inhibition of cell respiration is interpreted as a specific poisoning of the catalysts responsible for tissue oxidations, since effects of a similar nature are well known in many oxidation systems of both biological and non-biological origin.

Amongst the variety of animal and plant tissues studied, an inhibitory action of cyanide upon respiration has been observed in all but a few cases, and attention has been focussed almost entirely upon this effect. The extent of the inhibition appears to vary considerably according to both the kind and condition of the tissue, and at present the interpretation of these differences remains somewhat controversial. This aspect of the action of cyanide will not, however, be discussed in the present paper since we shall be concerned more particularly with an effect of cyanide which has not been recognised hitherto, although possible indications of its existence are to be found in the observations of other authors.

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In our experiments on the effects of hydrogen cyanide upon the potato tuber at 15° C., it is found that cyanide treatment produces a greatly increased respiration which may be maintained for periods of several weeks. This effect of cyanide stands in striking contrast to the marked inhibition of respiration, which has been observed in so many tissues, and which has come to be regarded as the dominant and usual effect of cyanide upon the metabolism of the cell.

This investigation further establishes that the increased respiration in cyanide is brought about not by a direct effect upon the oxidative system, but rather by a specific effect upon the starch-sugar relationships of the potato, resulting in an increased sugar concentration. In the potato at this temperature the specific poisoning of the respiratory enzymes does not become apparent except after a prolonged and drastic cyanide experience.

Among the observations in the literature a few examples are known in which cyanide produces relatively little or no inhibition upon the respiration, at least initially, *e.g.*, starved yeast, Warburg (1927), and Lathyrus seedlings, Genevois (1929). The only case for which there exists substantial evidence that cyanide causes an increase in respiration is that of *Chlorella* in the absence of added glucose: Warburg and Negelein (1919), Emerson (1927), and here the fact has elicited little attention beyond the suggestion by Warburg and Uyesugi (1924) that the increased respiration may be due to the utilisation of hydrogen cyanide as substrate for respiration. It is impossible to decide from the relatively few observations described in the literature, whether the increased respiration in *Chlorella* is of a similar nature to the effect, which will be described in the potato. This subject will be considered in a later paper.

II. METHODS.

In investigating the effects of cyanide upon the potato, continuous determinations were made of the respiration of samples of potatoes during exposures to atmospheres containing low controlled concentrations of hydrogen cyanide gas in air. In one type of experiment only the carbon dioxide production was measured; in a second type both carbon dioxide and oxygen consumption. In both types of experiment readings were continued day after day, usually for periods of several weeks, the aim being to establish the course of respiration during and after experiences in cyanide of different strengths and durations.

Measurement of Carbon Dioxide Production.

The method developed in the Botany School, Cambridge, was employed for measuring the carbon dioxide production. A stream of gas, freed from carbon dioxide, is drawn over the respiring material, and then bubbled through a Pettenkofer tube filled with standard baryta solution. The baryta tubes are replaced at intervals, and the residual baryta is determined by titration with standard acid.

The application of this method to studying the effects of hydrogen cyanide upon carbon dioxide production involved the solution of several technical difficulties. The requirements were :—

- (1) A constant known rate of the gas stream.
- (2) A device for generating hydrogen cyanide at a constant and controlled rate.
- (3) A method of collecting and estimating separately the carbon dioxide and hydrogen cyanide in the gas stream after leaving the respiration chamber.

The following methods were evolved to meet these requirements :—

(1) The gas stream was motivated by a constant negative pressure obtained from a filter pump acting through a mercury valve, and the rate of the stream was regulated by the limiting resistance of a length of capillary tubing. The rate in most experiments was 1500 c.c. per hour.

(2) A form of apparatus was developed which gave a controlled and approximately constant production of hydrogen cyanide. It consists essentially of a small reservoir of 25 per cent. sulphuric acid, into which a solution of potassium cyanide is allowed to drip at a slow uniform rate. The gas stream is bubbled through the acid, and carries out with it hydrogen cyanide gas. The acid in the generating chamber is drained out and replaced each day. The rate of entry of the potassium cyanide solution is kept constant by using a Mariotte's bottle as a reservoir for the solution, and restricting the flow by inserting a length of fine thermometer capillary tubing. By these means a constant rate of dripping was obtained, the rates varying in the different generators used, from 4 to 8 c.c. per day. By adjustment of the concentration of the potassium cyanide solution and the rate of the gas stream, any desired concentration of hydrogen cyanide could be obtained.

(3) A simple method was found for estimating both carbon dioxide and hydrogen cyanide in the gas stream after it left the respiration chamber. The stream was bubbled successively through two Pettenkofer tubes, the first

containing a standard solution of silver nitrate made slightly acid with nitric acid, the second a standard baryta solution. The hydrogen cyanide is absorbed quantitatively in the first tube, forming a precipitate of silver cyanide, and the amount is determined by estimating the residual silver ion by the Volhard method. The carbon dioxide is absorbed in the baryta tube, and its amount determined in the usual way by titration of the residual alkali with standard acid.

Measurement of Carbon Dioxide Production and Oxygen Uptake.

It will be sufficient to consider only the principle of the somewhat complicated apparatus which was designed to enable the carbon dioxide production and oxygen consumption to be measured continuously during treatment with hydrogen cyanide of known and controlled concentration. The apparatus was developed on a similar principle to that in use by Dr. Franklin Kidd, and modified to suit the requirements. A diagram of the apparatus is shown in fig. 1.

The compensating system, C, and the respiration circuit, R, H, P, N, G, are

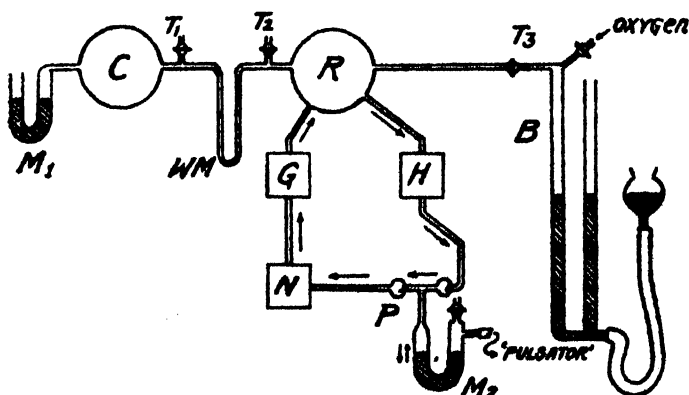


FIG. 1.—Diagram of the apparatus used for measuring the oxygen consumption and carbon dioxide production during treatment with hydrogen cyanide. For description see text.

two closed systems of equal gas volume, which are connected to the two limbs of a water manometer, WM. Each system includes a mercury manometer whose outer limb is open to the air. The two manometers (M_1 , M_2) are made of tubing of the same bore. In addition, each circuit may be opened to the atmosphere by the taps, T_1 and T_2 .

The respiring material is placed in R, and the atmosphere in this system is circulated in the direction indicated at a constant rate by means of the mercury

pump, P, of which the mercury column of manometer, M_2 , constitutes the piston, being made to oscillate by the intermittent suction device described by Blackman and Bolas (1927). When the outer limb is opened to the air the mercury column comes to rest, and M_2 becomes a manometer comparable to M_1 .

The hydrogen cyanide generator, G, is of the same general type as described above, but is so constructed that the volume of liquid in the acid reservoir may be drained accurately to a definite volume. The gas passes from G containing a constant concentration of hydrogen cyanide. It enters the respiration chamber, R, and from there is carried to H, a Pettenkofer tube filled with acid silver nitrate, in which the cyanide is absorbed. It then passes through the pump, P, to, C, a baryta tube which absorbs the carbon dioxide.

It will be seen then that hydrogen cyanide does not accumulate, but is being continuously generated in G and absorbed in H. Consequently, the volume of hydrogen cyanide in the system during a given cyanide treatment remains constant (and is actually a very small quantity). The carbon dioxide is also continuously absorbed in C as it is given off by the material, and consequently the only change in the amount of gas in the circuit is due to the oxygen utilised in respiration. This causes a reduction in pressure in the respiration circuit as compared with the compensating circuit, which leads to a difference in level on the two sides of the water manometer, WM.

At intervals the circulation is stopped by opening the outer limb of M_2 to the atmosphere. The various segments of the respiration circuit are then opened together through a system of capillary tubes (which are not shown in the diagram) and thus the pressures equalised throughout the circuit. The acid in the generator, G, is drained to the constant volume, and oxygen is then added to the circuit from the oxygen burette, B, through the tap, T_3 , until the pressures of the two systems are equalised as shown on the water manometer. The volumes of oxygen in the burette are measured at the existing atmospheric pressure, both before and after the addition of oxygen. The difference is the equivalent* of the oxygen consumed by the tissues, and its volume, temperature, and pressure are known. Moreover the oxygen concentration of the atmosphere in the respiration circuit is returned to its normal value.

The absorption tubes, C and H, are then removed and replaced, and fresh acid is run into the generator, G. Both the circuits are opened to the air, and then closed, and the circulation is once more started.

* The mercury manometers, M_1 and M_2 , being of the same bore, compensate for changes in barometric pressure between successive readings.

The carbon dioxide and the hydrogen cyanide are then determined as before by titrating the residual baryta and silver in the respective absorption tubes.

*Estimation of Sugar Content of Tissues.**

The samples to be analysed were cut rapidly into thin slices and frozen at -20°C . From this temperature they were removed for analysis as convenient. After grinding, the tissue was first boiled in alcohol, and then extracted in hot alcohol for 4 hours. The extracts were freed from alcohol by distillation under reduced pressure, and the residual liquid was cleared by treatment with basic lead acetate, excess lead being precipitated afterwards with potassium oxalate. The liquid was filtered and the clear solution was made up to a definite volume. The reducing power of this solution was determined by the method of Shaffer and Hartman, both before and after hydrolysis of sucrose, which was effected by boiling for 20 minutes in 10 per cent. citric acid. From these values the concentrations of both reducing sugars and sucrose in the sample of tissue are obtained, both values being expressed as invert sugar in percentages of fresh weight.

III. EXPERIMENTAL MATERIAL.

Potatoes of the King Edward variety were used for the experiments. A selected stock of small tubers varying in weight from 20 to 35 gm. was stored at 10°C ., and samples were removed to 15°C . as required.

At 15°C . the respiration rate falls slowly for about a month to a level which may be maintained for several months. The material is thus in a steady metabolic state which renders it particularly suitable for experimentation.

The respiration intensity of the potato is extremely low compared with most animal and plant tissues, and notwithstanding its solid appearance the structure is sufficiently porous to enable the whole of the tissue to respire aerobically under normal conditions. This is shown by the fact that the respiratory quotient is unity.

IV. CARBON DIOXIDE PRODUCTION DURING CYANIDE TREATMENT.

Experiment 1.—Samples of four to six potatoes were used to study the effects of different concentrations of hydrogen cyanide in air, and the records of carbon dioxide production† are given in fig. 2.

* We are indebted to the Hon. Mrs. Onslow for the supervision of the sugar analyses, and to Miss N. A. Potter who carried out the estimations.

† The carbon dioxide production is calculated as milligrams per hour per 100 grammes initial fresh weight of potato tissue.

The control sample, which was kept in air throughout, shows the typical respiration drift of an untreated potato at 15° C.

The four samples, A, B, C and D, after an initial period in air were exposed to atmospheres containing various low concentrations of hydrogen cyanide, during the periods indicated on each record. The concentration used for each sample is given; it varied from 0.14 c.c. hydrogen cyanide per litre in A, to 10 times that concentration in D.

The striking feature is that the rate of carbon dioxide production increases greatly in each case during the cyanide treatment, and that the increased respiration is not merely ephemeral, but of considerable duration.

The records for each sample show a similarity in form, there being two distinct phases during the cyanide experience, first a rise to a maximum, and then a downward drift. It is also evident that the magnitudes of the effects are closely related to the cyanide concentration; with increasing cyanide the slope of the phase of rising respiration is greater, the maximal value attained is higher, and the subsequent downward drift is more rapid.

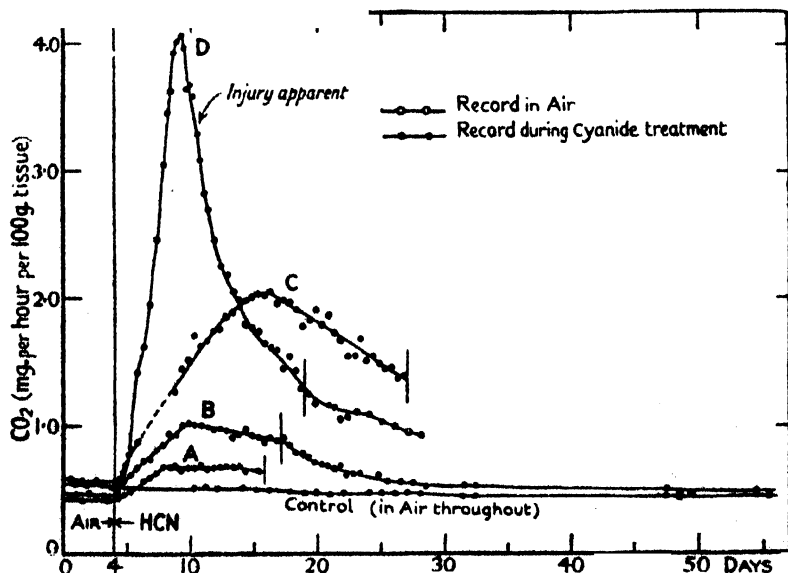


FIG. 2.—Experiment 1 (started November, 1920).—Effect on carbon dioxide production of exposure to atmospheres containing different concentrations of hydrogen cyanide. Duration of cyanide treatment is indicated in solid dots. The cyanide concentrations used were:—

Sample A—0.14 c.c. HCN per litre.

„ B—0.18 c.c. „

„ C—0.30 c.c. „

„ D—1.45 c.c. „

A distinction must be drawn, however, between the effects of treatment with the lower cyanide concentrations as in A, B, and C on the one hand, and the relatively much higher concentration of D. In the former the effects appear to be completely reversible, since on removal from cyanide the respiration falls slowly to about the control value and does not fall below it, and the potatoes remain sound for long periods. This is illustrated by the post-cyanide record of sample B (fig. 2). After removal to air the respiration fell gently, reaching the control level some 40 days later. Observations after 80 days in air—record not given—showed that this level was maintained, and the potatoes still appeared sound. At that time a second treatment with cyanide again resulted in an increase in respiration.

In sample D, on the other hand, signs of injury to the potatoes (brown patches and sunken areas) were evident early on the downward phase, *i.e.*, from the sixth day in cyanide. After the sample was removed to air, rapid breakdown occurred, and at the end of the record the potatoes were exuding liquid. Another indication of a difference in the case of D is that the downward drift in cyanide begins sooner, and at a lower pitch, than would be expected if record D were a member of the regular series suggested by the records A, B, and C.

This experiment serves to illustrate both the characteristic two-phase course, and the magnitude of the increased carbon dioxide production of the potato in cyanide over the range of concentrations used throughout the investigation. The effect of an appreciably stronger concentration than that used on sample D has not yet been tried.

It is convenient to mention at this point the question of diffusion of hydrogen cyanide into the potato. It is to be noticed in the records of fig. 2 that, when the samples are transferred to atmospheres containing cyanide, there is no indication of a prolonged lag in the effects upon respiration; the rate of increase of carbon dioxide production attains its maximal value within 24 hours in each of the three lower cyanide concentrations, and 36 hours in the high cyanide. Moreover, in converse experiments in which potatoes are removed from cyanide to air, it is found that the bulk of the cyanide given off is evolved in the first 24 hours. It would appear, therefore, that a state of equilibrium is attained relatively rapidly, and that the forms of the respiration records are not determined by changes in the internal concentration of cyanide.

V. OXYGEN CONSUMPTION AND CARBON DIOXIDE PRODUCTION DURING CYANIDE TREATMENT.

For the following experiments the apparatus previously described (p. 98) was used to obtain continuous measurements of both the oxygen consumption and the carbon dioxide production of samples of potatoes during a variety of cyanide treatments. The data are expressed in cubic centimetres of the gases at 0° C. and 760 mm. pressure, so that the units represent molecular equivalents of oxygen and carbon dioxide (1 c.c. carbon dioxide = 1.96 mg.).

Experiment 2.—The record of this experiment (fig. 3) shows the respiration rate (oxygen uptake and carbon dioxide production) of a sample of potatoes during successive periods in air, and in various cyanide-air atmospheres. In addition there is shown the carbon dioxide production of a control sample which remained in air throughout.

In the initial period in air the oxygen consumed is approximately equal to the carbon dioxide produced, and during the different cyanide experiences both values increase and decrease together. The rise in respiration in low cyanide (0.10 c.c. per litre), is followed by a fall on transference to air. There follows a period of 32 days in cyanide 0.29 c.c. per litre, and then the final period in cyanide 0.47 c.c. per litre.

During the long period in moderate cyanide, 0.29 c.c. per litre, the respira-

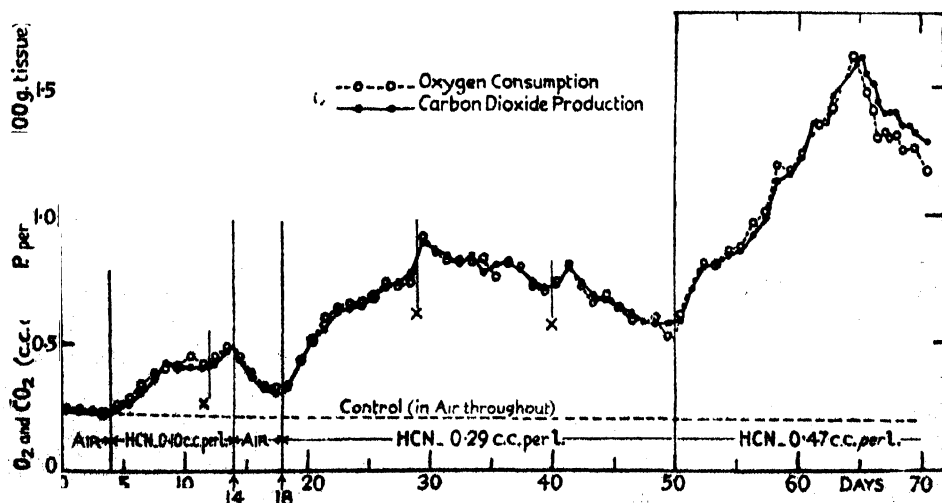


FIG. 3.—*Experiment 2* (started January, 1930).—Oxygen consumption and carbon dioxide production during a sequence of treatments with various cyanide concentrations. At the times marked X there were temporary increases in cyanide concentration due to slowing of circulation.

tion goes through the typical two-phase course, a rise to a maximum and a subsequent slower fall. The record over this period is actually nearly comparable in cyanide strength and in pitch to record C of experiment 1 (fig. 2).

In the final treatment the respiration rises fairly steeply to a value of 1.6, about eight times the control value, and only at this point, after 57 days in cyanide, is there a significant change in the respiratory quotient. It is evident then that profound changes in the respiration rate are induced by cyanide without affecting the respiratory quotient.

After the 65th day of the record, however, the oxygen values lie below the carbon dioxide. This increase in respiratory quotient is further investigated in the next two experiments.

Experiment 3.—A sample was treated with strong cyanide, 1.5 c.c. per litre, a concentration comparable with that of sample D of experiment 1. The oxygen uptake and the carbon dioxide production increase rapidly together

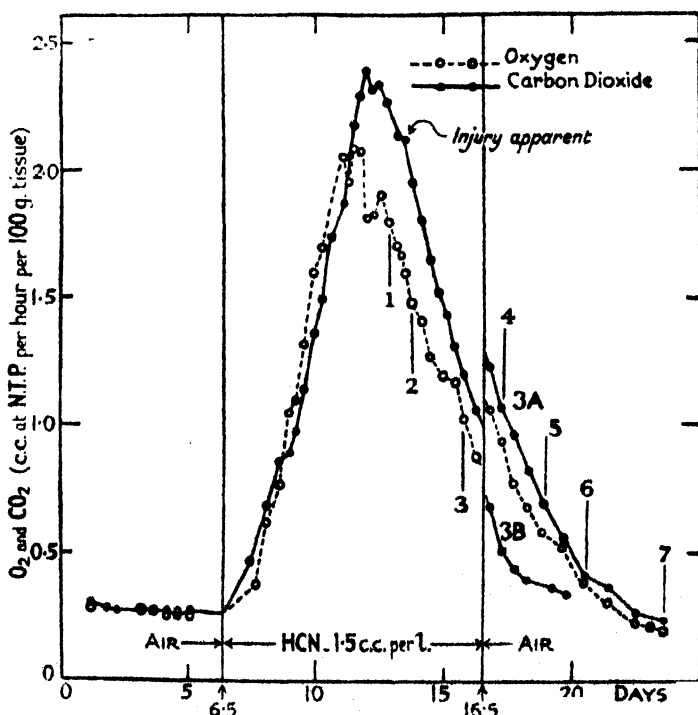


FIG. 4.—*Experiment 3* (started November, 1929).—Oxygen consumption and carbon dioxide consumption during treatment in high cyanide. After the 10 days' cyanide treatment the sample was divided into two parts, 3A and 3B (see text). The respiratory quotients at the times indicated (Nos. 1, 2, etc.) are:—

No.....	1	2	3	4	5	6	7
R.Q.	1.26	1.32	1.17	1.14	1.20	1.06	1.10

to a high value of about 2.1 (fig. 4) from which point onwards a marked change is evident, the oxygen values lying considerably below the carbon dioxide values throughout the subsequent part of the record. Brown patches of injured tissue developed shortly after the change in respiratory quotient.

After 10 days in cyanide the sample was divided into two parts, and returned to air. Readings of both oxygen and carbon dioxide were continued on the less-injured potatoes—3A, and it is seen that the high respiratory quotient was maintained during the subsequent fall in air. Only the carbon dioxide production was followed in the more severely injured potatoes—3B.

Experiment 4.—In order to investigate further the development of the increased respiratory quotient, a sample of potatoes was given alternate exposures to air and to relatively high cyanide in air (0.71 c.c. per litre) for the periods indicated in fig. 5.

A 4-day exposure to the cyanide atmosphere produces a rapid rise in respiration, which continues for 2 days after the sample is replaced in air, until a value of 2.0 is reached. During this rise, and the subsequent fall in air, there is no significant change in the respiratory quotient.

The second treatment in cyanide is of longer duration. A steep rise to approximately the same high level is again produced, but in this case when

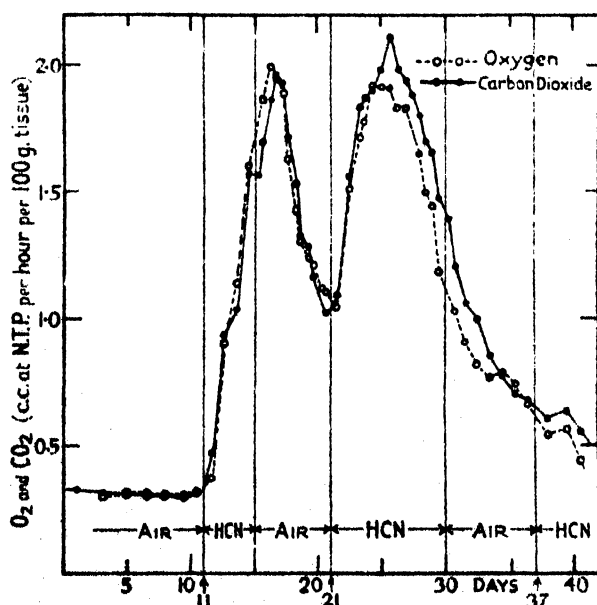


FIG. 5.—*Experiment 4* (started May, 1930).—Oxygen consumption and carbon dioxide production during alternating periods in air and relatively high cyanide—0.71 c.c. HCN per litre.

the respiration reaches 1.9, the respiratory quotient increases suddenly, and during the subsequent fall in cyanide, the oxygen values lie below the carbon dioxide.

It is evident then that the change in respiratory quotient is not merely a function of respiration intensity, but of duration and strength of the cyanide treatment.

On removal to air after the second cyanide treatment, the respiration continues the downward drift, the respiratory quotient at first remaining high, but later returning to approximately unity. This return to the normal respiratory quotient is in contrast to the effect found in experiment 3, fig. 4. where the cyanide treatment had been more severe before the removal to air, and the respiratory quotient remained high in air.

During the third and final cyanide treatment two points are noteworthy. First, the cyanide no longer causes an increase in the respiration rate, indicating that the previous treatment has produced an irreversible change in the metabolic system; second, the high respiratory quotient immediately returns.

In these three experiments two definite stages in the effects of cyanide upon respiration are distinguishable by the respiratory quotient. In the first stage the respiration may be changed considerably, but the respiratory quotient remains normal. For convenience we shall refer to treatments whose effects do not go beyond this stage as moderate cyanide experiences. The second stage is marked by the sudden development of an increased respiratory quotient which is followed immediately by a fall in the rate of respiration, and definite signs of injury. This stage is reached sooner in higher cyanide concentrations, but it is not yet known whether it might be produced by moderate cyanide after a sufficiently long exposure. Treatment leading to this stage will be referred to as severe.

VI. NITROGEN RESPIRATION AFTER CYANIDE TREATMENT.

It would appear from the previous experiments that the increased respiration induced by cyanide is of the same nature as normal aerobic respiration. Further evidence of this is derived from experiments in which potatoes, after cyanide treatment, are placed in nitrogen.

Experiment 5.—The record of sample A (fig. 6) shows the typical effect of nitrogen on untreated potatoes. The carbon dioxide production falls away in the course of 2 to 3 days to a value which is roughly 0.4 times the air line value. (There is sometimes a slight preliminary increase in carbon dioxide production preceding the fall, as in A, fig. 6.) On replacing in air there is a

striking after-effect, the carbon dioxide production rising for several days considerably above the air line.

The effects of nitrogen on cyanide-treated potatoes is shown in record B of fig. 6. Here a large sample of potatoes was kept in a low cyanide atmosphere

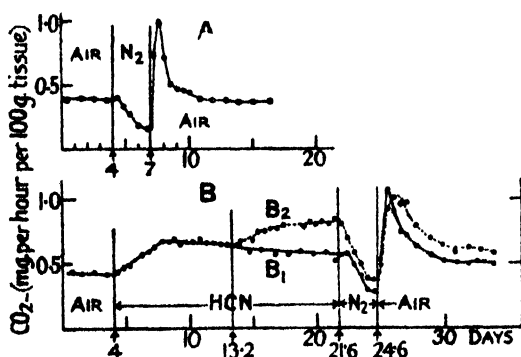


FIG. 6.—*Experiment 5* (started December, 1920).—Effects of nitrogen on carbon dioxide production, A—untreated potatoes, B—after cyanide treatment. (B in 0.14 c.c. HCN per litre, then divided into two parts, B₁ left in 0.14 c.c. HCN per litre, B₂ given 0.28 c.c.)

for 9 days and then divided into two lots, of which one, B₁, remained in the original cyanide concentration and the other, B₂, was exposed to double this concentration of cyanide, which is still a moderate strength. The respiration of the latter rose to a higher level. Both samples were removed from cyanide and placed in nitrogen for 4 days, and then in air. In each case the effect of this treatment is similar to that obtained with normal untreated potatoes, that is, a fall in nitrogen and a subsequent after-effect in air.*

VII. CHANGES IN SUGAR CONTENT DURING MODERATE CYANIDE TREATMENT.

Several lines of evidence suggested that the increased respiration induced by cyanide treatment is brought about by an increased concentration of the substrate of respiration. It is known, for example, that the respiration of the potato at 15° C. is limited to a large extent by the sugar concentration in the tissues, and that increases in respiration, similar in magnitude to those caused by cyanide treatment, can be produced if the sugar content is increased by

* The nitrogen levels reached in B₁ and B₂, respectively, are 0.49 and 0.46 times the corresponding air line values, ratios which are slightly higher than the value with the untreated potatoes. The possible significance of this difference must await further investigation. There is no doubt, meanwhile, of the general similarity of the nitrogen effects.

storage at low temperatures. Moreover, another line of evidence, which will be considered more fully later, suggested that cyanide might have a specific activating effect upon the amylase system, and so possibly bring about an increased production of sugar from reserves.

The sugar content of potatoes during cyanide treatment was, accordingly, investigated.

A.—Increase of Sugar during Rising Phase of Respiration.

Experiment 6.—In order to investigate the effect of cyanide upon the sugar content, a number of samples of potatoes, after various cyanide treatments, were analysed, together with untreated control samples. Sample A (fig. 7) was kept in cyanide of concentration 0.33 c.c. per litre for 14 days, during which time the respiration increased from 0.48 to 3.02. The sample was then analysed.

Five other samples, B, C, D, E, and F (of which E and F will be considered in the next section), were treated with a lower cyanide concentration (0.20 c.c. per litre), B being analysed after 7 days, C and D after 14 days in cyanide. The individual respiration records are shown in fig. 7. It will be noticed that there is considerable scatter in the records of these three samples, although the strength of cyanide was identical for each. Such variation is usual with small samples of 3 to 5 potatoes as in the present experiment.

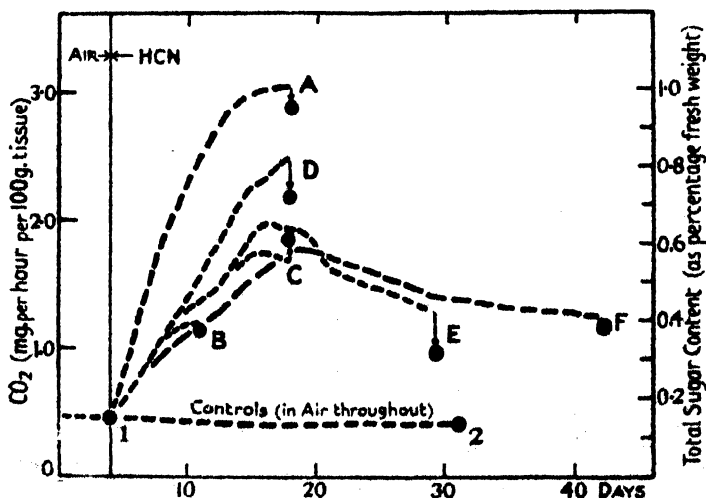


FIG. 7.—*Experiment 6* (started January, 1930).—Carbon dioxide production and sugar content during cyanide treatment. Sample A in 0.33 c.c. HCN per litre. Samples B, C, D, E, and F in 0.20 c.c. HCN per litre. Sugar content of each sample shown as ● at time of analysis.

The sugar content and the respiration values of samples A, B, C and D at the time of analysis are given in Table I. There are given in addition the values for two untreated samples, control 1, analysed at the beginning of the experiment, and control 2 at the end.

Table I.—Sugar Content and Respiration during Cyanide Treatment.

Sample.	Cyanide strength.	Days in cyanide.	Respiration when analysed.	Hexose.	Sucrose.	Total sugar.
	c.c. per litre			per cent.	per cent.	
Control 1	—	—	0.50	0.04	0.12	
Control 2	—	—	0.39	0.04	0.09	
Sample B	0.20	7	1.20	0.05	0.33	
Sample C	0.20	14	1.69	0.17	0.45	
Sample D	0.20	14	2.44	0.10	0.62	
Sample A	0.33	14	3.03	0.19	0.77	

The data show a striking increase in the sugar content of the treated potatoes, the amount of the increase depending both upon the cyanide strength and the duration of treatment. The accumulated sugar is largely sucrose, but the reducing sugar (hexoses) also increases.

When the respiration values are plotted against the sugar values as in fig. 8, the data suggest strongly that the respiration is proportional to the sugar

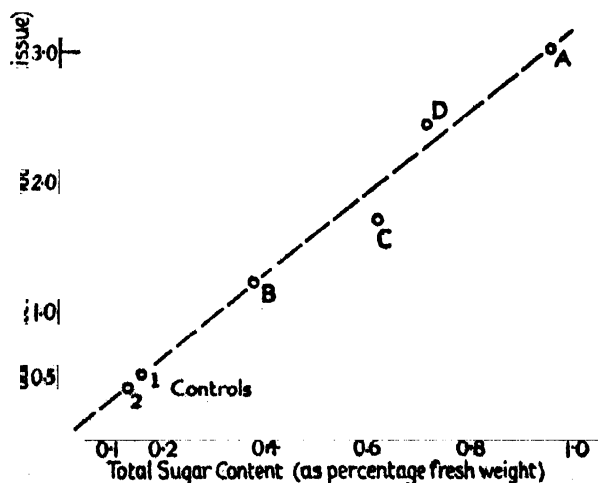


FIG. 8.—*Experiment 6.*—Relationship between respiration rate and sugar content during rising respiration phase.

content, over the range of sugar concerned. We may record here that an almost linear relationship over this range is also found in the case of potatoes whose sugar content is increased by low temperature.

B.—Decrease in Sugar during Falling Phase of Respiration.

To investigate the changes in sugar content during the falling respiration phase, the two samples, E and F, were left in the same cyanide strength (0.20 c.c. per litre) for 26 and 39 days, respectively. From fig. 7, in which the respiration records are plotted for the five samples, it can be seen that, whereas samples C and D were analysed approximately at the maximal respiration, the respiration of E and F had fallen considerably by the time of analysis. The divergence in the records of the two samples is again noticeable.

The sugar content and respiration values are given in Table II, together with those of samples C and D, for comparison.

Table II.—Sugar Content and Respiration during Cyanide Treatment.

Sample.	Duration of treatment.	Maximum respiration.	Respiration when analysed.	Hexose.	Sucrose.	Total sugar.
	days			per cent.	per cent.	per cent.
E	26	2.0	1.25	0.08	0.23	0.31
F	39	1.76	1.21	0.11	0.28	0.39
C	14	1.73	1.69	0.17	0.45	0.62
D	14	2.44	2.44	0.10	0.62	0.72

From this table it becomes evident that the sugar content of E and F has decreased rapidly during the falling respiration phase. Moreover the loss of sugar is more rapid in sample E, where the fall in respiration is also more rapid, since its sugar content on the 14th day of the falling phase is already lower than that of sample F on the 25th day.

The relationship between the sugar content and respiration is clearly shown in fig. 7, where the data for samples B to F throughout the cyanide experience are plotted.

C.—Decrease in Sugar after Removal from Cyanide.

Experiment 7.—An example of the fall in respiration after removal from cyanide to air after a moderate cyanide treatment, has already been given in experiment 1 (fig. 2), record B. After removal to air the respiration falls slowly to about the control value. The fall in air is more rapid when the cyanide treatment has been such as to induce higher respiration.

To ascertain the change in sugar content during the downward drift of respiration in air, two samples, G and H, were treated with the same cyanide concentration as sample A, experiment 6, i.e., 0.33 c.c. per litre.

The respiration record of sample G was very similar to that of A (fig. 7). At the time when A was analysed, after 14 days in cyanide, G was transferred to air. The respiration fell rapidly in the course of 4 days from 2.8 to 1.6, at which time the sample was analysed (fig. 9).

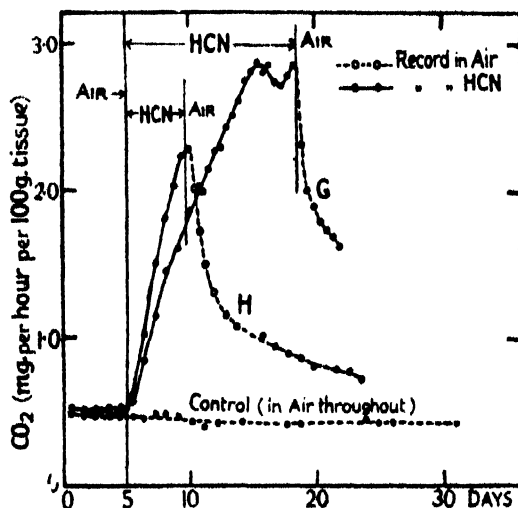


FIG. 9.—Experiment 7 (started January, 1930).—Carbon dioxide production during cyanide treatment (0.33 HCN per litre) and after removal to air. Samples were analysed at end of records.

Sample H remained in cyanide for 4½ days only, when its respiration had reached a value of 2.2. It was then placed in air, and the respiration fell to 0.7 in the course of 14½ days, when it was analysed. The respiration of the control at this time was 0.42 (fig. 9).

The sugar content and final respiration values of A, G, H, and the control are given in Table III. Comparing A and G it is evident that the sugar content decreased considerably during the fall in respiration in air. In H both the sugar content and the respiration were approaching the control level.

Table III.—Sugar Content and Respiration after Cyanide Treatment.

Sample.	Days in cyanide.	Days in air.	Respiration when analysed.	Hexose.	Sucrose.	Total sugar.
				per cent.	per cent.	per cent.
A	14	—	3.03	0.19	0.77	0.96
G	14	4	1.60	0.09	0.32	0.41
H	4.5	14.5	0.70	0.08	0.11	0.19
Control	—	—	0.42	0.04	0.09	0.13

VIII.—RELATIONSHIP BETWEEN RESPIRATION RATE AND SUGAR CONTENT.

The results of experiment 6 have shown that during cyanide treatment changes in the respiration rate are accompanied by corresponding changes in sugar content; during the rising phase of respiration the sugar content increases, during the falling phase it decreases. In experiment 7 the effects of removal from cyanide into air were investigated. Here again there are parallel changes in sugar content and respiration rates, both decreasing toward the normal value.

On the basis of this respiration and sugar data we may survey the ratio respiration/sugar content to see whether the respiratory efficiency is changed by the different cyanide experiences. In the first part of experiment 6 the relationship between respiration and sugar content during the rising phase in cyanide was shown to be approximately linear, as is indicated by the broken line of fig. 8. There is no evidence then of a changing respiratory efficiency during the rising phase in cyanide.

For purposes of comparison fig. 8 has been reproduced in fig. 10, where are plotted in addition the sugar and respiration values of the samples which were analysed (a) during the fall in cyanide—samples E and F, plotted as +, (b) during the fall in air after removal from cyanide—samples G and H, plotted as ●.

It will be seen that the points lie sufficiently close to the broken line to suggest that the respiratory efficiency is not appreciably altered during moderate cyanide experiences of this sort.

After more severe cyanide treatment, however, this relationship no longer holds. For example, the sugar and respiration values of sample 3B of experiment 3 are also plotted. In this case, the potatoes had undergone a prolonged treatment in a relatively high cyanide concentration; the respiratory quotient

had increased, and breakdown of the tissues had begun (fig. 4). The position of the point (3B in fig. 10) indicates a considerably diminished respiratory

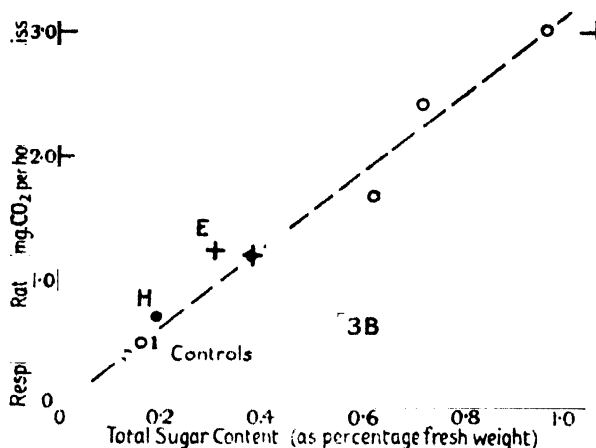


FIG. 10.—Relationship between respiration rate and sugar content. The broken line and circles—○, are plotted from fig. 8.

- during rising respiration phase.
- + after fall in cyanide.
- after fall in air.

efficiency since its respiration rate is only about one-third that of potatoes with the same sugar content on the rising respiration phase, as shown by the broken line in the graph.

IX. INTERPRETATION OF RESULTS.

A.—Respiration during Cyanide Treatment.

In the foregoing experiments a striking and hitherto unrecorded effect has been clearly defined. It has been shown that when potato tubers at 15° C. are exposed continuously to atmospheres containing low concentrations of hydrogen cyanide, the respiration increases considerably and may be maintained at a high level for periods of several weeks.

The respiration during a cyanide experience goes through a typical two-phase course, first a period in which the respiration rises to a maximum, and then a slower fall. Over the range of cyanide concentration investigated, the slopes of the two phases and the peak respiration values attained are greater with increasing cyanide.

The effects of moderate cyanide experiences, on the one hand, and relatively

severe cyanide experiences, on the other, exhibit important differences. In the experiments which have been described three metabolic states are distinguishable during cyanide treatment, and these will be made the basis of a preliminary analysis of the cyanide effects.

Stage A.—A phase of rising respiration in which the respiratory quotient remains unchanged. The respiration may rise to as much as six to eight times the normal value. This stage is produced by all strengths of cyanide investigated.

Stage B.—A phase of falling respiration in which the respiratory quotient remains unchanged. This occurs in moderate cyanide experiences, following stage A.

Stage C.—A phase of falling respiration of which the onset is marked by a sudden increase in the respiratory quotient to a value of 1.1 to 1.25. This stage occurs only in the severe cyanide experiences, and it follows immediately after stage A.

It will be noticed that in stages A and B the respiration appears to be similar in nature to normal aerobic respiration, since the respiratory quotient remains unchanged. These stages, moreover, seem to be completely reversible. In stage C, on the other hand, not only is the respiratory quotient increased, but permanent injury to the tissues develops, and subsequent cyanide treatment after removal to air no longer induces increased respiration. With moderate cyanide treatment the sequence of effects is stage A—stage B; with high cyanide stage A—stage C. It is not yet clear whether a sufficiently prolonged experience in moderate cyanide might induce the sequence stage A—B—C, but so far this has not been observed.

B.—Sugar Changes in Moderate Cyanide.

The experiments, in which sugar analyses were made, have shown that the drifts in respiration during both the rising phase (stage A) and the falling phase in cyanide (stage B) (that is during the two phases of unchanged respiratory quotient) are accompanied by parallel drifts in the sugar content of the potatoes. Moreover it has been established that the respiratory efficiency (the ratio respiration/sugar concentration) is not appreciably changed throughout such an experience, showing that there is no apparent effect upon the respiratory enzymes.

As already mentioned, there is good reason to believe that sugar concentration forms a limiting factor in the normal respiration of the potato at 15° C.—a large body of evidence in favour of this view has arisen in a detailed study

of the effects upon respiration of increasing the sugar concentration by storage at low temperatures. The results of the present investigation also support this view that the rate of respiration at 15° C. is conditioned by the sugar concentration.

The changes in respiration rate induced by cyanide would appear, therefore, to be due, not to a direct effect of cyanide upon the catalytic systems responsible for respiration, but to an effect of cyanide upon the starch-sugar relationships resulting in changed sugar concentrations in the tissues. According to this view, then, the increased respiration in cyanide is to be regarded as an effect produced by an increased substrate for respiration.

This hypothesis, and, indeed, the whole of this investigation, was developed in close relationship with an investigation of the properties of certain amylase preparations *in vitro*. The results of this study have so important a bearing on the interpretation of the present experiments that it is thought essential to include here a brief outline of the main features of this work.

C.—Cyanide Effects on Barley Amylase in vitro.

In an investigation of the effects of cyanide upon a large number of preparations of barley amylase, several interesting phenomena have come to light. It has been found that under certain conditions cyanide exerts a dual action upon the enzyme.

There is first a rapid activation, after which a slower inactivating process becomes apparent, which obscures the activation and leads eventually to the partial, or in some cases, total destruction of the enzyme. This inactivating process is greatly retarded by the presence of the substrate, starch, or by low temperature.

The magnitude of the activation varies from one preparation to another,* and also according to the previous history of the enzyme. For a particular preparation the degree of activation depends upon the cyanide concentration, and the relationship appears to be hyperbolic.

An activated amylase, under conditions where the inactivating process is negligible, becomes deactivated by the removal of the cyanide as hydrogen cyanide under reduced pressure.

* Some preparations have been found which are not activated by cyanide ; in other cases, the activation may be extremely great, as much, for example, as 25 times the control value. A full discussion of the problems connected with the incidence and character of the activation will be given in a subsequent paper.

D.—*Cyanide Effects on Starch-Sugar Relationships of the Potato.*

The above effects of cyanide upon certain amylase preparations *in vitro* suggest a basis for interpreting the changes in sugar content in the potato during cyanide treatment.

When potatoes are treated with cyanide at 15° C. the sugar concentration (and the respiration) increase progressively for a time and at a rate which depends upon the cyanide concentration (stage A). It is evident that during this phase there is an increased hydrolysis of reserve carbohydrates. It is suggested that this is due to an activation of the starch-hydrolysing system of the potato, corresponding to the activation of amylase *in vitro* by cyanide.

Eventually this period of increased sugar production comes to an end, and the metabolic system settles down to a new drift (stage B) in which the sugar content and respiration are falling. During this phase the rate of production of sugar from reserves is no longer sufficient to maintain the former high sugar level. It would appear that a second effect of cyanide has now come into play, an inactivation of the system which was originally activated. This would correspond to the second effect observed *in vitro*, the inactivation.

Removal into air after a moderate cyanide experience causes a drift of the sugar content, and respiration, back toward the normal state. This suggests that the cyanide-activated mechanism becomes "deactivated" by the loss of hydrogen cyanide, an effect which again has a strict parallel in the case of amylase preparations *in vitro*.

It will be seen, then, that the effects of cyanide upon the starch-sugar relationships in the living potato suggest a close analogy with the effects, which have been demonstrated on amylase preparations *in vitro*.

The close analogy between these effects tends to support the hypothesis that cyanide produces changes in the starch-hydrolytic system similar to those observed *in vitro* with preparations of barley amylase. According to this view it would seem that the rate of production of sugar in the potato is determined by the activity of the amylase system.

The further development of this hypothesis must await an extension of the study on amylase extracts, and, in particular, an investigation of the effects of cyanide upon the amylase of the potato.* This work, and also further experi-

* We note in the 'Amer. J. Bot.,' vol. 17, p. 1050, Dec., 1930, that F. E. Denny, with whom we discussed the progress of our work at the International Botanical Congress, 1930, has since then investigated this particular point. Denny confirms our expectation, namely, that extracts of potato amylase can be activated by cyanide *in vitro*.

ments on changes in sugar content in potatoes treated with cyanide, are in progress.

E.—Inhibition of the Respiratory Enzymes.

We have seen that in stage B, the falling phase in moderate cyanide, the decreasing respiration is due to a falling sugar concentration and not to an inhibition of the respiratory enzymes. During the rapid fall in high cyanide (stage C), however, in addition to the changed respiratory quotient, we find a marked decrease in the respiratory efficiency. In this stage of cyanide treatment the inhibition of the respiratory enzymes becomes apparent, that is, the effect which is usually associated with the physiological action of cyanide.

Further investigation of this aspect of cyanide action is in progress.

F.—Cyanide Effects upon Cell Mechanism—Limiting and “Non-limiting” Factors.

We have seen that the various effects of cyanide upon the metabolic system become apparent in a definite sequence. It is evident that the order of appearance, and the magnitudes, of the different effects will depend upon the relationships of the individual processes affected to the system as a whole. It seems clear that where the rate of respiration is largely limited by one particular part of the metabolic system, then any effect of cyanide upon this “limiting factor” will preponderate over effects upon other cell mechanisms which under the particular conditions are “non-limiting.” In such a system cyanide may act upon several cell mechanisms, but specific effects on “non-limiting factors” may be almost completely obscured, until these in turn become “limiting.”

Consequently, in the case of the potato, although there is no direct evidence that the oxidative systems of the cells are being inactivated during a moderate cyanide experience, yet it cannot be concluded that this effect is absent, since under the conditions of our experiments the rate of respiration is limited by the sugar concentration. Under such conditions relatively large changes in the effective amount of respiratory enzyme might result in negligible changes in respiration rate.

It is felt necessary to emphasise this point of view since it has been found in the case of the potato, that the nature of the cyanide effect depends upon the metabolic state of the tissues. The present investigation has established that at 15° C. cyanide induces an increase in the respiration rate, and that this is due to a specific effect upon the starch-sugar relationships. We shall show in a subsequent paper that, in other metabolic states, cyanide effects upon other cell mechanisms become apparent.

X. SUMMARY.

(1) The respiration of potato tubers at 15° C. increases when they are exposed to atmospheres containing low concentrations of hydrogen cyanide.

(2) The respiration rate goes through a typical two-phase course, first a rise to a maximum, and then a slower fall.

(3) During moderate cyanide treatment the respiratory quotient remains unchanged and the effects are reversible. During severe treatment, however, the onset of the falling phase is marked by an increased respiratory quotient, and irreversible injury occurs.

(4) During the rising phase of respiration in cyanide the sugar content increases. In moderate cyanide the falling respiration phase is accompanied by a parallel fall in sugar content, and there is no evidence of inactivation of respiratory enzymes.

(5) The changes in respiration rate in a moderate cyanide experience are due to changes in the concentration of sugar.

(6) The changes in sugar concentration are interpreted as a direct effect of cyanide upon the starch-hydrolysing system of the potato, since analogous effects of cyanide have been observed with certain amylase extracts *in vitro*.

(7) During the falling respiration phase in high cyanide, in addition to the development of an increased respiratory quotient, inactivation of the respiratory enzymes is shown by the decreased respiratory efficiency (the ratio respiration rate/sugar concentration).

We wish to thank Dr. F. F. Blackman for his stimulating interest throughout the investigation, and Dr. D. Keilin for helpful criticism. We are indebted to Mr. Arthur E. Porter for assistance in the experimental work.

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*The Antiseptic and Trypanocidal Action of certain Styryl and Anil Benzthiazole Derivatives.**

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In view of the powerful antiseptic action of certain anil quinoline compounds (Browning, Cohen, Ellingworth and Gulbransen, 1926) and the trypanocidal action of these and especially of some styryl quinoline derivatives (Browning, Cohen, Ellingworth and Gulbransen, 1929), it was thought desirable to examine the action of similar compounds containing other heterocyclic nuclei. The present communication is concerned with a number of derivatives of benzthiazole, in which the pyridine ring of the quinoline group is replaced by a five-membered ring containing both nitrogen and sulphur.

Antiseptic Action.

Antiseptic power was estimated as in previous communications (Browning, Cohen, Ellingworth and Gulbransen, 1926, 1928). Throughout the range of the anil benzthiazole compounds examined the antiseptic activity is considerably lower than that of the corresponding quinoline compounds. The styryl compounds tend also to be weak in their antiseptic action and in this respect resemble the styryl quinoline series, but in general they are not inferior to the latter in potency. Frequently in the present series of compounds the irregularities in action, previously commented upon,† were observed in marked degree.

Comparison of Styryls and Anils.—In the quinoline series, the anils were distinctly more potent than the corresponding styryls, especially against *B. coli*. The styryl and anil benzthiazoles, on the other hand, do not exhibit any marked contrast. In several cases (cf. 349, 336; 253, 252; 363, 362;

* The work reported in this communication was done with the support of the Medical Research Council.

† Browning, Cohen, Gaunt and Gulbransen, 'Proc. Roy. Soc.,' B, vol. 93, p. 329 (1922).

365, 364; 330, 329) the styryl compound is slightly more potent against staphylococcus in peptone water than is the corresponding anil.

Effect of Serum.—The action in the presence of serum appears to differ according to the organism used. Whereas in most cases the action on staphylococcus is greatly reduced by the presence of serum, there is in general a tendency towards increased potency against *B. coli* in serum when compared with peptone water.

Substitution in the Benzene Nucleus.—In the styryl group, the relative effects of a primary, secondary, tertiary or acetylamino group are such as would be expected in view of the effect of similar changes in the styryl quinolines. Thus, the amino styryl (254) is less active than the dimethylamino compound 253, and substance 332 (amino) is slightly less potent than either 349 (methylamino) or 330 (dimethylamino). In the anil series there is little difference in activity between the primary, secondary and tertiary compounds (*cf.* 335, 336 and 329). The acetylamino styryl compounds 368 and 333, as in the case of the corresponding quinoline derivatives, have little or no antiseptic action; but No. 377, like its quinoline analogue No. 90, has more pronounced action on staphylococcus. The acetylamino anil derived from nitroso tetrahydroquinoline (338) has also a powerful action on staphylococcus in peptone water.

Substitution in the Benzthiazole Nucleus.—In the previous communications regarding the quinoline compounds, it was observed that in general an increase in the mass of the quinoline nucleus tended to increase the antiseptic power. The same tendency was shown to some extent in the benzthiazoles. Thus, the α - and β -naphthathiazole derivatives 363, 365 (styryl) and 362, 364 (anil) are rather more active than the corresponding benzthiazole compounds 253 and 252.

In the anil compounds the introduction of an acetylamino group, however, did not increase the antiseptic action (252, 329); whereas in the corresponding substances of the anil quinoline series this was a marked feature. The chlor-acetyl anil derivatives (340, 341) appeared to be practically equal to the acetyl compound 329.

It should be noted that no attempt has been made to prepare a complete series of derivatives for comparison of antiseptic action, since it became evident at an early stage of the work that the group as a whole was greatly inferior to the anil quinoline compounds in this respect. Later, compounds were prepared solely with a view to investigating trypanocidal action.

Trypanocidal Action.

The benzthiazole derivatives, both anil and styryl, exhibited trypanocidal action when appropriate groups were present in the respective nuclei. This effect was tested for as before, in mice infected with *T. brucei*, treatment being administered 24 hours after inoculation, when scanty parasites were present in the blood. The necessary groups proved to be identical with those which produced trypanocidal action in the quinoline series. Thus the maximum effect was produced when one nucleus contained a basic group, and the other an acetylamino group. As in the quinoline series, the styryls were much more powerful and definite in action than the anils.

Cures were obtained with the following compounds which contain substituent groups as shown :—

Styryl No.	Group in benzene nucleus.	Group in benzthiazole nucleus.
332	NH ₂	NH . COCH ₃
349	NH . CH ₃	NH . COCH ₃
330	N(CH ₃) ₂	NH . COCH ₃
368	NH . COCH ₃	NH ₂
333	NH . COCH ₃	NH . COCH ₃
377	NH . COCH ₃	N(CH ₃) ₂

In the anil benzthiazole group, some action was observed with all the compounds except No. 252, but only with No. 336 was cure obtained. A striking feature of No. 329 was the tendency for therapeutic action to occur on a wide range of dosage (1/150 to 1/4000), although even the largest dose failed to produce cure.

In the quinoline series, the anil possessing the strongest trypanocidal action was that containing a dimethylamino group in the benzene nucleus, and a chloracetyl amino group in the quinoline nucleus. No cure was observed with the corresponding benzthiazole derivatives 340 and 341.

Ber azo]

No.	Substance.	Antiseptic action.										Precipitation.		Trypanocidal action.		
		<i>Staphylococcus aureus</i> .					<i>B. coli</i> .									
		Peptone water.		Serum.			Peptone water.		Serum.			In peptone water.	In serum.	Dose.	Result.	
		+	±	-	+	±	-	+	±	-	+					±
335	2 (p-amino anil) acetylaminio benzthiazole . Me ₂ SO ₄	40	20	4	10	4	2	10	4	2	40	20	10	2	1/2000	Slight
336	2 (p-methylaminio anil) acetylaminio benzthiazole . Me ₂ SO ₄	200	40	20	40	20	10	2	—	1	200	100	40	2	1/2000 1/4000	(Cure) Trace
352	2 (p-dimethylaminio anil) benzthiazole . MeCl	200	100	40	20	—	10	1	—	?	2	—	1	4	1/2500	0
362	2 (p-dimethylaminio anil) α-naphthathiazole . Me ₂ SO ₄	400	200	100	100	40	20	10	2	1	40	20	4	—	1/800	Trace
364	2 (p-dimethylaminio anil) β-naphthathiazole . Me ₂ SO ₄	1000	400	200	400	200	20	100	40	4	100	40	10	—	1/200	Trace
339	2 (p-dimethylaminio anil) acetylaminio benzthiazole . Me ₂ SO ₄	100	40	1	40	20	?	1	—	?	20	10	?	2	1/150 1/500 1/4000	Marked Slight
341	2 (p-dimethylaminio anil) chloroacetylaminio benzthiazole . MeCl	40	20	1	4	—	2	1	—	?	10	—	4	10	1/200	Slight

340	2 (p-dimethylamino anil) chloroacetylamino benzthiazole . Me_2SO_4	40	20	1	10	—	4	2	1	?	10	—	4	20	—	1/200	Slight
338	Condensation product of nitroso tetrahydro quinoline and acetylamino benzthiazole . Me_2SO_4	400	—	200	100	40	20	40	—	20	40	20	10	4	—	1/1500	Trace

Antisepic action.—In this and the following table the numbers are the reciprocals $\div 1000$, of the concentrations which produce the effects shown in 48 hours at 37° C.

+ = free growth of the organisms with the development of marked turbidity of the medium.

± = inhibition of growth, the medium being only faintly turbid or remaining unclouded, but in the latter case sub-cultures yielding growth.

— = complete sterility as tested by the absence of growth in sub-cultures.

Precipitation.—The numbers are the reciprocals $\div 1000$, of the lowest concentrations which cause precipitation in the media.

Trypanocidal action.—The doses shown are reckoned per 20 gram body-weight of the mouse. The highest dose shown for each substance is one which approximates to the largest amount borne by uninfected animals without producing obvious toxic effects, e.g., loss of weight.

The terms used to designate the lesser degrees of trypanocidal action are as follows :—

Trace = prolongation of life for several days beyond that of the untreated controls.

Slight = disappearance of parasites from the blood for several days to a week.

Marked = absence of parasites from the blood for 10 days or longer.

(Cure) indicates that with the doses shown cure was effected only in a proportion of the animals treated.

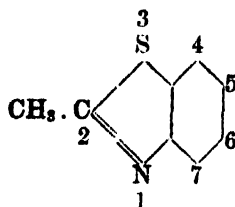
Styryl Benzothiazoles.

No.	Substance.	Antiseptic action.										Precipitation.		Trypanocidal action.	
		<i>Staphylococcus aureus</i> .					<i>B. coli</i> .								
		Peptone water.		Serum.			Peptone water.		Serum.			In peptone water.	In Serum.	Dose.	Result.
		+	±	+	±	—	+	±	—	+	±				
264	2 (p-amino styryl) benethiasole . MeCl.....	200	100	20	10	—	4	20	10	4	4	—	2	1/10000	0
332	2 (p-amino styryl) acetylaminobenthiasole . Me ₂ SO ₄	40	20	10	40	20	10	1	—	?	10	4	?	1/1500 1/5000 1/10000	(Cure) Slight
349	2 (p-methylamino styryl) acetylaminobenthiasole . Me ₂ SO ₄	200	—	100	20	—	10	10	—	4	10	—	4	1/1500 1/2500 1/5000 1/7500 1/10000	Cure (Cure) Slight
263	2 (p-dimethylamino styryl) benethiasole . MeCl.....	400	200	100	40	20	10	100	—	40	40	—	20	1/10000	0
343	2 (p-dimethylamino styryl) α-naphthathiasole . Me ₂ SO ₄	?	400	200	200	100	40	100	40	2	20	10	4	1/2000	Trace
345	2 (p-dimethylamino styryl) β-naphthathiasole . Me ₂ SO ₄	2000	—	1000	200	100	40	20	10	4	40	20	10	1/2500	Trace

330	2 (p-dimethylamino styryl) acetyl- amino benzthiazole . Me ₂ SO ₄	200 100	40	40	—	20	1	—	?	20	10	4	100	1	1/1500 1/5000 1/7500	(Cure) Trace
333	2 (p-acetylamino styryl) amino benz- thiazole . MeCl	2	1	?	4	3	1	—	?	10	4	1	1	—	1/1000 1/5000 1/7500 1/10000	Cure Marked
333	2 (p-acetylamino styryl) acetylamino benzthiazole . Me ₂ SO ₄	1	—	?	4	2	1	—	?	1	—	?	2	—	1/800 1/1500 1/5000	Cure Trace
377	2 (p-acetylamino styryl) dimethyl- amino benzthiazole . MeCl	400 200	100	20	—	10	1	—	?	10	4	1	—	—	1/1000 1/2000 1/2500 1/5000 1/10000	Cure Marked

CHEMICAL SECTION.

The following system of numbering has been used :—



2-Methyl benzthiazole was made according to the method of Jacobson ('Ber., vol. 19, p. 1072 (1886)'). It was found difficult to obtain a uniform yield in different preparations, much apparently depending upon the rate of melting of the mixtures of acetanilide and phosphorus pentasulphide. It was essential that these reagents should be thoroughly well ground up together, and after solution of the product in alcohol and subsequent dilution, it was necessary to regulate fairly accurately the addition of alkali in order to allow precipitation of the crystalline by-product, and to obtain the main product in solid form.

The thio-acetanilide, purified as described, was almost colourless and melted sharply at 76°. The maximum yield obtained was 51 per cent. of the weight of acetanilide taken, compared with 25 to 30 per cent. claimed by Jacobson. This is equivalent to 45.6 per cent. of the theoretical.

The oxidation of the thioacetanilide was carried out as described by Jacobson, the same yield being obtained.

2-Methyl benzthiazole methochloride.—Equivalent quantities of the base and dimethyl sulphate were mixed and warmed gently on the water-bath. Vigorous combination soon occurred, and a dark viscid mass was formed, which soon crystallised. It was converted to the methochloride by boiling the aqueous solution with hydrochloric acid and barium chloride. The filtered solution was evaporated to dryness and the product extracted with absolute alcohol, from which, on concentration, the required methochloride separated in almost colourless crystals.

Amino 2-methyl benzthiazole.—The nitro derivatives was first prepared by the direct nitration of 2-methyl benzthiazole. 2-methyl benzthiazole (14.9 gm.) was dissolved with cooling in 45 c.c. concentrated sulphuric acid. A mixture of 14.9 c.c. concentrated nitric acid (sp. gr. 1.42) and an equal volume of concentrated sulphuric acid was added gradually, the mixture being cooled in ice water and the temperature kept below 10°. After standing for 20

minutes at room temperature the mixture was poured into a large volume of ice water. The sulphate of the nitro compound, which separated, was filtered off, mixed into a cream with water, and made alkaline with ammonia. The product was recrystallised from alcohol, and formed colourless needles, melting at 165–166°. The yield was 12 gm. equivalent to 61·8 per cent. of the theory.

0·1510 gm. gave 19·4 c.c. N at 18° and 747 mm. N = 14·69 per cent.

$C_8H_6O_2N_2S$ requires N = 14·43.

The amino derivative was obtained by reduction of the nitro compound in the ordinary way with stannous chloride and hydrochloric acid. It was recrystallised from water, and formed long colourless needles melting at 124–125°. The yield on reduction was 76 per cent. of the theory.

0·3197 gm. gave 48·4 c.c. N at 19° and 751 mm. N = 17·24 per cent.

$C_8H_8N_2S$ requires N = 17·08 per cent.

Acetyl amino 2-methyl benzthiazole was obtained by heating the amino compound on the water bath with half its weight of fused sodium acetate and excess of acetic anhydride. The diluted mixture was made alkaline with ammonia, and the product filtered off and recrystallised from boiling water, with the addition of a little charcoal. It formed colourless prisms melting at 149–150°.

0·3041 gm. gave 35·4 c.c. N at 11° and 750 mm. N = 13·71 per cent.

$C_{10}H_{10}ON_2S$ requires N = 13·59 per cent.

Acetylamino 2-methyl benzthiazole methosulphate was prepared by heating the base in nitrobenzene solution with a slight excess of dimethyl sulphate at 100°. The resulting product was filtered, and thoroughly washed with ether. It was then used directly for condensation. On crystallisation from alcohol it yielded colourless crystals.

0·3236 gm. gave 23·5 c.c. N at 12·5° and 746 mm. N = 8·45 per cent.

$C_{13}H_{13}O_2N_2S_2$ requires N = 8·43 per cent.

Acetylamino 2-methyl benzthiazole methochloride, obtained as in the case of the corresponding quinaldine derivative, was salted out from the concentrated aqueous solution of the methosulphate with sodium chloride.

Amino 2-methyl benzthiazole methochloride.—The acetylamino methochloride, prepared as above, was boiled for 1 hour with concentrated hydrochloric acid. The solution was cooled, diluted, neutralised with ammonia,

and saturated with sodium chloride. The amino methochloride crystallised on standing, and was filtered, pressed, and dried. It was used for condensation without further purification.

Dimethyl amino 2-methyl benzthiazole methiodide.—An attempt was first made to prepare this compound from amino 2-methyl benzthiazole in one operation, as described in the former paper for 6-dimethyl amino quinaldine. The amino 2-methyl benzthiazole (1 mol.) was boiled with an aqueous solution of sodium carbonate (1 mol.) and methyl *p*-toluene sulphonate (3 mols.). Instead of a clear solution, however, an oil, partly soluble in the hot solution, was formed. After cooling, this oil was extracted with chloroform, the extract dried, and the solvent removed. The residual oil solidified in a freezing mixture, but melted again on warming to room temperature. All attempts to crystallise it were unsuccessful. The product did not contain iodine, and it appeared that the amino group had been methylated (since CO_2 was evolved vigorously during the preparation) without the formation of the quaternary group.

The product was finally obtained by heating the oil described above with excess of methyl iodide in toluene or alcoholic solution in a sealed tube for 3 to 5 hours at 100 to 110°. The resulting crystalline deposit was filtered off and recrystallised from aqueous alcohol containing some sulphur dioxide (to remove any periodide)*. Small yellow crystals were obtained, which on drying at 100° *in vacuo*, became colourless owing to loss of water of crystallisation.

0.1614 gm. gave 0.1144 gm. AgI, I = 38.32 per cent.

$\text{C}_{11}\text{H}_{18}\text{N}_2\text{SI}$ requires I = 38.00 per cent.

Anil benzthiazole Derivatives.—These were all prepared by condensation of the benzthiazole quaternary derivative in alcoholic or aqueous alcoholic solution with the appropriate nitroso compound. They are very similar in appearance to the corresponding quinoline compounds, and crystallised from the alcoholic solution in prisms showing a blue or bluish green reflex. They form blue or blue-violet solutions in alcohol, the aqueous solutions being of a redder shade. Similar compounds have been previously described by Smith (1923), and Hamer (1929).

No piperidine was necessary to effect these condensations.

Styryl benzthiazole Derivatives.—These compounds were obtained in the

* In the absence of sulphur dioxide the iodine content was too high.

same way as the anils, the nitroso compound being replaced by the appropriate aldehyde. Piperidine was in all cases used to accelerate the reaction.

The products, as in the case of the quinoline compounds, gave redder solutions, both in alcohol and water, than the corresponding anils.

2 (*p*-Acetylamino styryl) amino benzthiazole methochloride separated from the alcoholic solution in scarlet needles (or flat prisms) depending upon the temperature of separation).

0.2719 gm. (dried at 100° *in vacuo*) gave 26.9 c.c. N at 16.5° and 774 mm. N = 11.76 per cent.

$C_{18}H_{18}ON_2SCl$ requires N = 11.68 per cent.

2 (*p*-Acetylamino styryl) acetylamino benzthiazole methosulphate crystallised from the boiling alcoholic solution, in which it was prepared, in orange plates or flat prisms. It formed an orange solution in water.

2 (*p*-Acetylamino styryl) dimethylamino benzthiazole methochloride.—Dimethylamino benzthiazole was obtained from the amino compound by the method described above. The methiodide (1.1 gm.) was dissolved in alcohol and heated, if necessary, to remove sulphur dioxide and *p*-acetylamino benzaldehyde (0.6 gm.) added with three drops of piperidine. After boiling for 3½ hours the product was filtered hot, dried and analysed.

0.2423 gm. gave 18.7 c.c. N at 18° and 751 mm. N = 8.84 per cent.

$C_{20}H_{22}ON_2SI$ requires N = 8.77 per cent.

It was converted into the methochloride by boiling the methyl alcohol solution with silver chloride and evaporating to dryness after filtering off the silver iodide.

We are indebted to Dr. K. E. Cooper for the preparation of considerable quantities of methylbenzthiazole, its nitro and amino derivatives.

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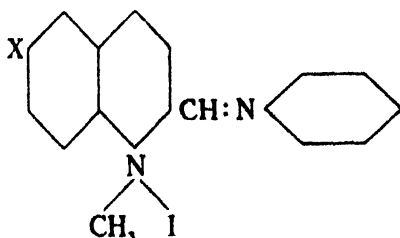
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*Some Aliphatic and Aromatic Amino Derivatives of α -Quinoline Methiodide.**

By J. B. COHEN, F.R.S., K. E. COOPER, and P. G. MARSHALL, Medical School, Leeds.

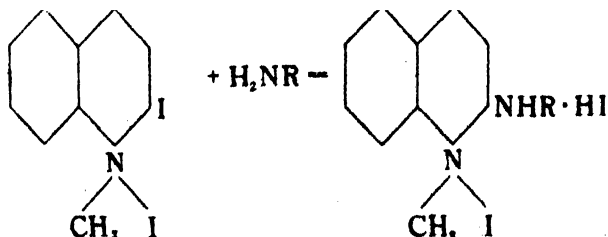
(Received January 2, 1931.)

In a series of papers by Browning, Cohen, Ellingworth and Gulbransen it was shown that many of the amino and acylamino compounds obtained by the condensation of derivatives of α -methylquinoline with nitroso-acylamines possess active antiseptic, and in some cases mild trypanocidal properties. These substances have the following general formula in which X represents an amino or acylamino group.



It appeared interesting to examine the physiological effect of substances obtained by attaching by means of nitrogen a basic aliphatic or aromatic side-chain directly to the α -carbon of the quinoline nucleus, replacing in this way the above CH group. These substances are in general easily obtained from the methiodide of α -iodoquinoline by the direct action of the base or by using as condensing agent pyridine or piperidine according to the following reaction.

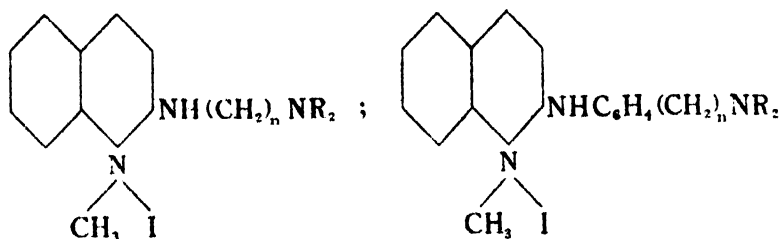
The action of alkalis on these substances removes hydrogen iodide and an imino-compound results.



* The work reported in this communication is done with the support of the Medical Research Council.

A number of these substances were submitted to Professor Browning of the University of Glasgow, who found that they exhibited no marked antiseptic or trypanocidal character.

Similar reactions have been carried out with diamino compounds of the aliphatic and aromatic series with basic groups at both ends of the chain of the following general formula,



in the hope that such substances might exhibit antimalarial action, but no such effect was observed.

As these substances have not been previously described, we give the following brief summary of the results obtained.

EXPERIMENTAL.

Iodoquinoline Methiodide.—This preparation was obtained by the oxidation of quinoline methiodide to N-methyl 2-quinolone (Decker, 1893). The quinolone was converted into 2-chloro quinoline, b.p. 272–278° (Perkin and Robinson, 1913), and the latter into 2-iodoquinoline methiodide by heating with excess of methyl iodide in a sealed tube at 100° (Roser, 1894). A better yield can be obtained by refluxing the chloro-compound with excess of methyl iodide for 8 days (Hamer, 1928).

6-Methoxy 2-iodoquinoline methiodide p-quinanisol was prepared by Skraup's synthesis from anisidine (D.R.P. 28,324). It distils at 163–170°/20 mm. The 6-methoxy N-methyl 2-quinolone was obtained from the corresponding methiodide by oxidation at 45–50° as described above. It crystallises from petroleum ether in colourless needles, m.p. 80°. It was converted into 6-methoxy 2-chloroquinoline as in the case of 2-chloroquinoline (see above) and crystallised from alcohol, petroleum ether or benzene in colourless plates, m.p. 107–108°. Found Cl, 18.76; $\text{C}_9\text{H}_8\text{NCl}$ requires Cl, 18.35 per cent.

6-Methoxy 2-iodoquinoline methiodide was obtained by heating the chloroquinoline with methyl iodide at 100° for 36 hours. It forms bright yellow crystals. Found I, 60.1; $\text{C}_{11}\text{H}_{11}\text{NOI}_2$ requires I, 59.5 per cent.

Diethylamino-ethylamine was prepared as follows:— β -bromethyl phthalimide (20 grams) (Gabriel, 1889) was heated in a sealed tube with diethylamine (11.5 grams) for 6 hours at 110° . The product was hydrolysed by boiling with five times the weight of 20 per cent. hydrochloric acid for 2 to 3 hours. The mixture was left overnight, the phthalic acid filtered and the filtrate evaporated as far as possible on the steam bath. Powdered potassium hydroxide (15 grams) was then stirred in with a pestle and the mixture extracted with benzene which dissolved the base. The benzene solution after dehydration over solid potassium hydroxide was distilled. The base boiled at $75^{\circ}/20$ mm. and yielded 2 grams of a colourless liquid with a strong ammoniacal smell which fumed in the air.

Diethylamino-propylamine was obtained by a similar method to the above by heating γ -bromopropyl phthalimide (18 grams) with diethylamine (11 grams) in a sealed tube for 6 hours at 110° . The base was separated as described above and yielded 2.5 grams of a pure, colourless liquid which fumed in the air and boiled at $90^{\circ}/20$ mm.

p-Amino benzyldiethylamine was prepared by mixing *p*-nitrobenzylbromide (9 grams) with diethylamine (3 grams) dissolved in a little alcohol. A vigorous action occurs which was completed by heating on the steam bath for half an hour. The product was dissolved in dilute hydrochloric acid, filtered from insoluble matter through a fluted filter, the solution made alkaline and extracted with ether. The ether after dehydration was removed and yielded 7.5 grams. The nitro-compound (7.5 grams) was mixed with alcohol (19 c.c.) and tin (6.3 grams) and concentrated hydrochloric acid (38 c.c.) slowly added. When the action had moderated, the mixture was heated on the steam bath until the liquid gave a clear solution with water. The base was extracted in the usual way. It distilled at $177\text{--}178^{\circ}/20$ mm. as a colourless liquid; the yield was 5.5 grams.

p-Amino-phenylethyldiethylamine.—Phenyl ethyl bromide (13.5 grams) prepared from phenyl ethyl alcohol was mixed with diethylamine (10.5 grams) in a sealed tube and heated in a steam bath for 6 hours. The product was dissolved in dilute hydrochloric acid and unchanged bromide removed (3 grams). The solution yielded 7.5 grams of base, b.p. $120\text{--}122^{\circ}/20$ mm. The base, 7.5 grams, was dropped slowly into fuming nitric acid (30 c.c., sp. gr. 1.5) kept at -10° to -5° and then poured on to ice (Barger, 1909). The base was precipitated by alkali and extracted in the usual way with ether. It yielded 8.8 grams of a pale yellow nitro-compound. The latter was reduced with tin and hydrochloric acid and gave a colourless liquid base (7.4 grams), b.p. $170\text{--}175^{\circ}/20$ mm.

Compounds of Iodoquinoline Methiodide with Monamines.

The following compounds were obtained by boiling the iodo-compound with a slight excess of an alcoholic solution of ammonia or the base for $\frac{1}{2}$ –1 hour.

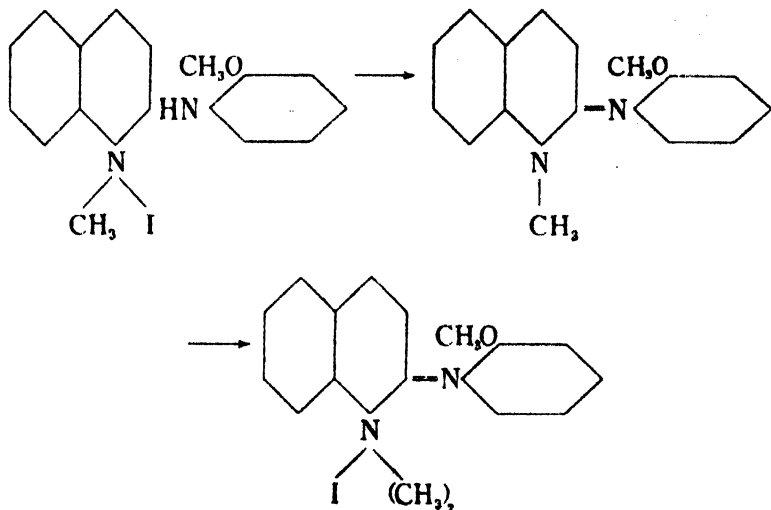
2-Aminoquinoline methiodide by the action of alcoholic ammonia. It crystallises from alcohol in long yellow needles, m.p. 250–251°. Roser (1894) gives 247°.

2-Dimethylamino-quinoline methiodide by the action of dimethylamine. It crystallises from alcohol in yellow needles, m.p. 199–200° (Roser, *ibid.*, gives 197°).

2-Diethylamino-quinoline methiodide by the action of diethylamine. It crystallises from alcohol in yellow needles, m.p. 202° (decomp.). Found, I, 36.9; $C_{14}H_{19}N_2I$ requires I, 37.2 per cent.

2-(p-Anisylamino)-quinoline methiodide by the action of *p*-anisidine in 60 per cent. alcohol. It crystallises from water in pale buff needles, m.p. 230–231°. Found, I, 32.1; $C_{17}H_{17}ON_2I$ requires I, 32.4 per cent. Alcoholic ammonia converts it into a halogen-free base, m.p. 114–115°, crystallising in yellow needles from dilute alcohol. It combines with methyl iodide, giving a methiodide which crystallises in golden yellow plates from water and dilute alcohol, m.p. 185° (decomp.). Found, I, 31.7; $C_{18}H_{19}ON_2I$ requires I, 31.2 per cent.

2-(o-Anisylamino)-quinoline methiodide by the action of *o*-anisidine in 60 per cent. alcohol. It crystallises in pale yellow needles from water, m.p. 224–225°. Found, I, 32.55; $C_{17}H_{17}ON_2I$ requires I, 32.4 per cent. Alcoholic ammonia forms the halogen-free base, which is soluble in ether and crystallises in yellow needles, m.p. 95–96°. With methyl iodide it yields a methiodide which crystallises from alcohol in needles, m.p. 192° (decomp.). Found, I, 30.93; $C_{18}H_{19}ON_2I$ requires I, 31.2 per cent. These compounds probably lose hydrogen iodide with ammonia and then unite with methyl iodide as follows :—



2-Piperidyl quinoline methiodide was prepared by the action of piperidine. It crystallises from alcohol in yellow needles, m.p. 195° . Found, I, 35.96; $C_{18}H_{19}N_2I$ requires I, 35.90 per cent.

2-(6-Amino quinaldine) quinoline methiodide was prepared by the action of 6-amino-quinaldine. Crystallised from water and then from alcohol, it forms small orange plates, m.p. 170° . Found, I, 29.4; $C_{20}H_{18}N_3I$ requires I, 29.7 per cent.

Compounds of Iodoquinoline and 6-Methoxy-iodoquinoline with Diamines.

Ethylene diamine bis-quinoline methiodide was obtained by the action of ethylene diamine. It was crystallised first from alcohol and then boiled with acetic anhydride for 3 hours, precipitated with water and finally crystallised from water. It gave colourless crystals, m.p. $286-288^{\circ}$. Found: I, 42.4; N, 9.5; $C_{22}H_{24}N_4I_2$ requires I, 42.5; N, 9.4 per cent.

2-(γ -Diethylamino β -hydroxypropylamino) quinoline methiodide was formed by the action of $NH_2 \cdot CH_2CH(OH) \cdot CH_2N(C_2H_5)_2$ (prepared from epichlorhydrin and diethylamine, D.R.P. 473,219). It was crystallised from alcohol. It forms microscopic, colourless crystals. Found: I, 46.3; $C_{17}H_{27}ON_3I$ requires I, 46.8 per cent. By boiling with alcoholic ammonia a base was obtained, m.p. 180° . Found: I, 30.8; $C_{17}H_{28}ON_3I$ requires I, 30.6 per cent. The first product is probably



2-Diethylamino-ethylamino quinoline methiodide.—Diethylamino-ethylamine and iodoquinoline methiodide in molecular proportions were mixed and stirred, when heat is evolved. The mixture was then heated for half an hour on the steam bath. The product was dissolved in a little boiling water, filtered from any unchanged methiodide and cooled. The filtrate deposited clusters of flattened prisms of the hydriodide having a faint pink colour, m.p. 241° (decomp.). Found: I, 49.1; N, 8.3; $C_{16}H_{24}N_3I_2$ requires I, 49.6; N, 8.2 per cent.

2-Diethylamino-ethylamino-6-methoxy quinoline methiodide.—Methoxyiodoquinoline methiodide (1 gram), diethylamino ethylamine (0.6 gram) and pyridine (1 gram) were heated on the steam bath for quarter of an hour and on cooling, excess of dilute hydriodic acid was added. The solution was filtered hot and on cooling the hydriodide of the base separated in fine needles with a faint pink colour, m.p. $203-204^{\circ}$. Found: I, 46.9; $C_{17}H_{23}N_3I_2$ requires I, 46.8 per cent.

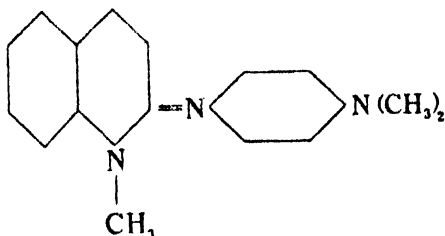
2-Diethylamino-propylamino-quinoline methiodide.—Diethylaminopropylamine dissolved in twice its volume of benzene was added to three times its weight of the iodoquinoline methiodide. The mixture became hot. It was then heated on the steam bath for a quarter of an hour and the product dissolved in boiling water, filtered from any iodoquinoline methiodide and the crystalline hydriodide recrystallised from water. It forms flattened prisms with a faint pink colour, m.p. 216° (decomp.). Found: N, 8.25; $C_{17}H_{23}N_3I_2$ requires N, 8.0 per cent.

2-Diethylamino-propylamino-6-methoxy quinoline methiodide.—The compound was prepared in the same way as the diethylaminoethylamino derivative (see above) using equal weights of the base, methoxyquinoline methiodide and pyridine. The product crystallises in needles from alcohol having a pale yellow colour, m.p. $229-230^{\circ}$. Found: I, 45.6; $C_{18}H_{25}N_3I_2$ requires I, 45.6 per cent.

2-(p-Amino-phenylamino) quinoline dihydrochloride was obtained by the action of *p*-aminoacetanilide followed by hydrolysis with alcoholic hydrogen chloride. It crystallises in colourless leaflets, m.p. about 270° (decomp.). Found: Cl, 23.22; $C_{13}H_{15}N_3Cl_2$ requires Cl, 23.05 per cent.

2-(p-Dimethylamino-phenylamino) quinoline methiodide was obtained by the action of dimethyl *p*-phenylene diamine in 60 per cent. alcohol solution. The hydriodide crystallises in yellow brown needles. Addition of pyridine to an alcoholic solution of the hydriodide removes hydriodic acid, giving the methiodide which crystallises in red brown needles, m.p. 241° (decomp.). Found: I, 31.88; $C_{18}H_{20}N_3I$ requires I, 31.36 per cent.

The hydrochloride obtained by the addition of alcoholic hydrogen chloride to the base in alcohol has a greenish tint and dissolves readily in water. It melts with decomposition at about 205° . Alcoholic ammonia yields after a few minutes boiling a halogen-free base, which dissolves in ether and crystallises in needles, m.p. 129.5° . It is probably represented by the formula :



On heating the base with methyl iodide under pressure a methiodide is obtained in yellow-brown needles from water, m.p. $223-224^{\circ}$ (decomp.). Found : I, 30.9 ; $C_{19}H_{22}N_3I$ requires I, 30.3 per cent.

2-(*p*-Amino-benzyl-diethylamino) quinoline methiodide.—Iodoquinoline methiodide (2 grams) and the base (1 gram) dissolved in a little benzene were mixed and stirred and heated for half an hour on the steam bath. The product was dissolved in hot water, filtered and crystallised. The hydriodide separates in yellow prisms with rounded ends, m.p. 241° . Found : I, 44.9 ; N, 7.14, $C_{21}H_{27}N_3I_2$ requires I, 44.2 ; N, 7.31 per cent.

2-(*p*-Amino-benzyl-diethylamino) 6-methoxy-quinoline methiodide.—Methoxy-quinoline methiodide (1 gram) *p*-aminobenzyl-diethylamine (0.5 gram) and pyridine (1 gram) were heated for not more than a quarter of an hour on the steam bath and stirred. The product which had a dark brown colour was treated with cold absolute alcohol, filtered and washed with alcohol. The light brown crystalline precipitate remaining on the filter was crystallised from dilute hydrochloric acid. The hydrochloride forms clusters of prisms of a light buff colour. The yield was 0.5 gram, m.p. 245° (decomp.). Found : 1 gram = 0.7451 mixed silver halides ; theory, 1 gram = 0.7372.

2-(*p*-Amino-phenylethyl-diethylamino) quinoline methiodide.—Iodoquinoline methiodide (1 gram) *p*-aminophenyl-ethyl-diethylamine (0.5 gram) pyridine (1 gram) in 10 c.c. absolute alcohol were mixed and left at the ordinary temperature for 24 hours. It turned dark red. The product was dissolved in hot alcohol with addition of a little hydrochloric acid. Clusters of fine needles of a deep red colour separated, which were filtered and washed. On dissolving the red needles in water, crystals of a rhombohedral shape and buff colour

separated. The substance is apparently the hydriodide of the base. Found : I, 43.4 ; $C_{23}H_{29}N_2I_2$ requires I, 43.1 per cent.

2-(*p*-Amino phenylethyldiethylamino) 6-methoxy-quinoline methiodide.—Methoxy-iodoquinoline methiodide (1 gram), aminophenylethyl diethylamine (0.5 gram) and pyridine (2 grams) were mixed and kept at 45° for 16 hours. The product was filtered, washed with absolute alcohol and the residue dissolved in the smallest quantity of hot, dilute hydrochloric acid. Clusters of brown, needle-shaped crystals separated, which, after drying *in vacuo*, melted at 260° (decomp.). Like the above, the product (it is the hydriodide for the same product) was obtained when it was crystallised from hydriodic acid in place of hydrochloric acid. Found : I, 40.6 ; $C_{23}H_{31}ON_2I_2$ requires 41.0 per cent.

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The Reproductive Processes of Certain Mammals. Part I.—The Œstrous Cycle of the Chinese Hamster (Cricetulus griseus).

By A. S. PARKES, Foulerton Student of the Royal Society.

(Communicated by C. L. EVANS, F.R.S.—Received January 22, 1931.)

[PLATES 5-7.]

I. *Introduction.*

The rapid growth of experimental work on reproduction has tended to obscure the fact that our knowledge of the foundation of the subject, *i.e.*, of the comparative morphology of the reproductive processes, is still remarkably meagre. Detailed information as to the morphology of the changes occurring during the Œstrous cycle is available for less than a dozen species, of which the mouse, rat, guinea-pig, rabbit, ferret, dog, pig and cow are best known. Some inadequate data on sheep and horses and one or two Marsupials and Primates are also available, but the total is insignificant compared with the wealth of material available even in a country with a restricted fauna (see Marshall, 1922, and Parkes, 1929). At the moment, for instance, we are almost completely ignorant of the cycle of even the domestic cat. Of the species for which information is available, only two (the rat and mouse) fail to differ from every other in well-recognised features, and there is little reason to doubt that the study of further species will reveal new types and variations. At the moment it seems clear that the experimental work of the last few years is rapidly outgrowing its foundations, which have been but little added to during this period.

It seemed essential, therefore, that efforts should be made to increase the range of our knowledge of the comparative morphology of the Œstrous cycle, and the present paper constitutes the first of a series designed to record the results of work in this direction.

II. *Material and Technique.*

Cricetulus griseus, the Chinese hamster, is a native of China and Eastern Asia generally, and is a nocturnal rodent closely related to the rat and mouse. Some notes on fertility and pre-natal death have been made by Fortuyn (1929, 1929a, 1930).

The animals used in this work were given by Professor E. Hindle to Professor J. P. Hill, who very kindly passed them on to me. Two males and 10 females, weighing between 25 and 35 gm. and all apparently adult, were received. Of the females, H3 was kept under observation for some weeks but is not available for histological description owing to sudden death, while H2, H7 and H9 showed no signs of cyclic changes and their ovaries were subsequently found to be in an advanced stage of fatty degeneration. A fifth hamster (H6) underwent cyclic changes in the vagina, but the ovaries also showed extreme fatty degeneration, and were useless. Two stages of the ovarian cycle were obtained from most of the remaining animals by means of a preliminary unilateral ovariectomy. The operations were carried out as previously described for mice.

Vaginal smearing was performed as usual, once a day as a routine, but every 8 hours during the time when the exact length and nature of the cycle was being studied, and also preparatory to killing the animals. The smears were stained with Leishman's compound or safranin. Ovaries, uteri and vagina were fixed in Bouin's fluid as a routine, two ovaries only being fixed in Flemming's fluid. Complete serial sections were cut of all ovaries.

Table of Available Material.

No. of animal.	Stage of cycle.	Material available.
H5	Pro-oestrus	Right ovary, uterus and vagina obtained at autopsy.
H8	Oestrus (early)	Left ovary obtained by operation.
H8	" (mid)	Right ovary, uterus and vagina obtained at autopsy.
H10	" "	Both ovaries, uterus and vagina obtained at autopsy.
H4	" (late)	" " " " " "
H5	Met-oestrus	Left ovary obtained by operation.
H1	Dioestrus (early)	Right ovary, uterus and vagina obtained at autopsy.
H1	" (mid)	Left ovary obtained by operation.

III. General Morphology of the Reproductive Organs.

The reproductive organs of the Chinese hamster are remarkably similar to those of the rat and mouse. The ovaries are bean-shaped, and completely enclosed within a fibrous capsule developed from the peritoneum. The Fallopian tube is long, but much coiled, as in the mouse. The uterus is completely bicornuate, the cornua fusing just above the cervix. There are two distinct cervical canals through the greater part of the cervix, but these join and form a single canal immediately before entering the vagina. As in the

mouse, the clitoris is large and distinct from the vaginal orifice. The urethra runs down along the vagina and finally emerges through the clitoris—there is thus no uro-genital sinus in the hamster. The mammary glands are distributed over the thorax, abdomen and inguinal region, 4 to 5 pairs being the usual number.

The male hamster is remarkable for the large size of the testes and accessory glands, which have been described in the European species by Grosz (1905).

IV. *General Features of the Cycle.*

Although the two males showed spermatogenesis and appeared to be perfectly normal on autopsy, while half of the females at least had normal oestrous cycles, no copulation was observed and no breeding took place. Attempts at mating invariably resulted in a violent fight and defeat of the male, one of which, not being rescued soon enough, was killed by the female. No explanation of this antagonism was forthcoming, since it was apparent when the female was in the stage of ovulation, as well as during dioestrus. Judging by the cyclic changes in the females, no restricted season of sexual activity occurred.

Owing to the failure to induce copulation, it was not possible to make observations on pregnancy, pseudo-pregnancy or lactation. Professor J. P. Hill, however, has allowed me to look over sections of some hamster uteri sent to him from China. These uteri were originally thought to contain embryos, but histological examination showed that the nodules in the uteri were not caused by embryos, but were due to the presence at fairly regular intervals on the mesometrical side, of large glandular-looking bodies between the muscular layer of the uterine wall and the peritoneal covering. These bodies are very similar in appearance to corpora lutea, and are almost certainly secretory in nature. They appear to constitute definite myometrial glands, such as have been alleged by Bouin and Ancel to occur during the latter half of pregnancy in the rabbit. In these hamster uteri, however, the glands are perfectly definite and obvious, their average maximum diameter being about 1.5 to 2.0 mm. As many as 8 to 10 of these bodies occur in one cornu (see Plate 5, fig. 1). Since the uteri in question contained no embryos and since similar glands were not found in the animals described in this paper, it is not easy to explain their occurrence in these uteri belonging to Professor Hill. It is possible, however, that the glands develop during pregnancy and atrophy only slowly during lactation, when they would be found in the non-pregnant uterus.

In four animals completely free from signs of fatty degeneration, 32 follicles

due to ovulate at the next œstrus were found, representing an average of four per ovary. Judged by this criterion, the size of litter in the Chinese hamster is not materially different from that of the mouse.

The Nature of the Œstrous Cycle.—While information as to the complete sexual cycle has not been available, fairly adequate data have been obtained on the nature and length of the normal œstrous cycle in the unmated female.

Since associated changes in the vagina have proved an invaluable guide to the ovarian cycle in related rodents such as the mouse, it was anticipated that examination of the vaginal contents of the hamsters would reveal cyclic changes of a similar kind. In practice, examination soon showed that the vaginal epithelium underwent cyclic changes of a type very similar to those found in the mouse and rat. As in these animals, the most striking phase in the vaginal cycle is that characterised by the occurrence of large blocks of cornified epithelial cells in the smear. This phase was subsequently found to correspond, as in the rat and mouse, with the maturation and ovulation of the Graafian follicle. Distention of the uterus at this time, as found in the mouse, also occurred in the hamster. The length of the cycle in the hamster is very similar to that in the mouse, the average time from ovulation to ovulation in the unmated condition being a little under 5 days.

Length of the Œstrous Cycle.—Analysis of the vaginal changes, which were found to be diagnostic of the ovarian cycle, showed that variation in the length of cycles in the five normal hamsters available was not appreciable. Table I shows the individual histories of the animals.

Table I.—Length of Cycle in Individual Unmated Hamsters.

No. of hamster.	Length of cycles.	Total cycles.	Average length.
	days.		days
H1	5-4-4-4-4-4-4-4-4-3-3-6	13	4.2
H3	5-4-5-4-4-6-7-3-6-3	10	4.7
H4	3-3-4-6-6-4-4-4-3-3-4	11	4.0
H5	3-4-4-4-5-4-4-5-4-3-4-4-5	14	4.0
H8	4-7-5-5-4-4-4-4-5-5-4-4-4-4-5-4-7-4-4-4	21	4.5

The average length of cycle is thus 4.3 days. The frequency distribution for length of cycle is given in Table II.

Table II.—Frequency Distribution of Length of Cycles in Unmated Hamster.

	3	4	5	6	7	Total.
Length of cycle in days	3	4	5	6	7	
Number of cycles	11	38	11	6	3	69

For this series $\sigma^2 = 0.96$ and the mean length of cycle is thus 4.3 ± 0.079 .

These records include all the cycles observed, except five erratic cycles in H6, which was subsequently found to have ovaries in an advanced stage of fatty degeneration and which is not considered in the present paper.

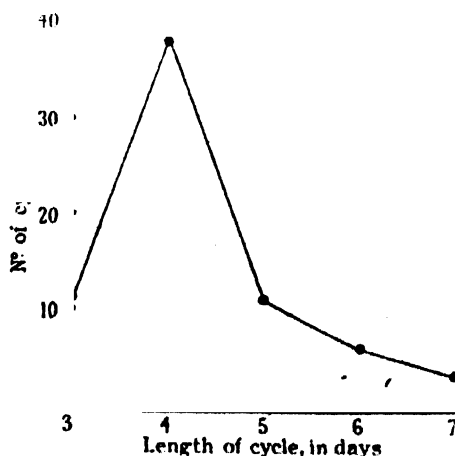


FIG. 1.—Frequency polygon for length of oestrous cycle in unmated hamster.

V. Cyclic Changes in the Ovaries, Uterus and Vagina.

Vaginal Cycle.—The vaginal cycle, initially investigated by examination of the nature of the vaginal contents, is very similar, both in nature and in the duration of the phases, to that occurring in the mouse. The chief difference is that in the hamster the changes are even more definite than in the mouse; the stage in which the vaginal contents consist solely of nucleated epithelium being remarkably prominent. The longest phase is, of course, that in which large numbers of leucocytes associated with some nucleated epithelial cells make up the vaginal contents. This phase lasts some 2 to 3 days, and is superseded by one in which the vaginal contents consist solely of nucleated epithelial cells. This stage is of short duration, usually less than 1 day. The nucleated cells are gradually superseded by cornified epithelial cells, which are sloughed off from the vaginal epithelium coincidently with the final growth

of the Graafian follicle in the ovary. The stage of cornification lasts $1\frac{1}{2}$ to 2 days, ovulation taking place towards the end of the phase. In one or two unusual cycles, appreciable quantities of nucleated cells appeared in the vaginal smear towards the end of the cornified stage. This may be accounted for by supposing that in such cases very severe desquamation of the epithelium took place. Leucocytes then return, followed by nucleated epithelial cells, the cornified cells disappear, and the smears return to the interval type.

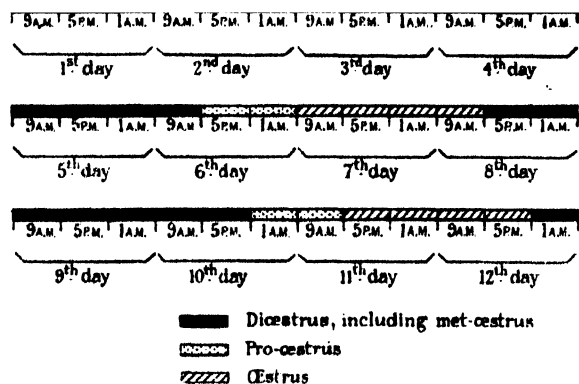


FIG. 2.—Vaginal smear sequence in Hamster 5.

Illustrations of the various types of smear are given in Plate 7, figs. 1 to 3. In view of this close correspondence with the cycle in the rat and the mouse it seems quite legitimate to apply the same terms, i.e., dioestrus, pro-oestrus and oestrus to the successive phases. The reservation should be made, however, that since no copulation was observed in these animals, an important, if not the most important, criterion of the oestrus phase is missing. Fig. 2 shows the actual sequence of events and their time relations through three cycles of H5.

On a basis of the data given in Section IV and of analysis of the phases of the cycle, the following average figures may be given for the length of the cycle and of its phases :—

	Days.
Total cycle	4½
Pro-estrus	½
Estrus	1½
Metestrus	} 2½
Dicestrus .	

The Ovarian Cycle.—The growth curves of the Graafian follicles and of the corpora lutea through the cycle are shown in fig. 3. The follicles due to ovulate

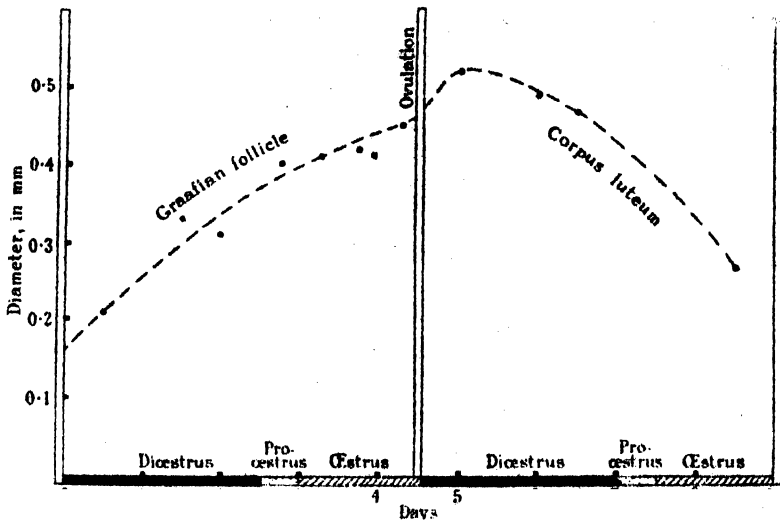


FIG. 3.—The ovarian cycle in the hamster.

at any given oestrus only become distinguishable from the group of small follicles after the preceding oestrus. Growth appears to be fairly steady right up to the time of ovulation, which occurs at the very end of the phase characterised by cornification of the vagina. During the growth preceding ovulation, the follicle more than doubles its diameter, increasing from about 0.2 mm. to over 0.4 mm. This represents an increase of about 10 times in volume. No follicle in the course of ovulation, or immediately after was obtained, but the time of ovulation can be judged from the fact that the latest specimen obtained during the stage of vaginal cornification possessed the largest follicles observed, while the earliest post-oestrous specimen (12 hours after the reappearance of leucocytes) showed young hollow corpora lutea. These corpora lutea were larger than the largest follicle obtained. The size of the corpora lutea is maintained during the second day of dicoestrus, after which a rapid decrease occurs. By the time of the next oestrus, the corpora lutea from the preceding ovulation are so small and shrunken as to be hardly detectable. This rapid decline in size of the corpora lutea is in striking contrast to the mouse and rat, in which as many as three or four sets of corpora lutea, representing different ovulations, can be seen in the ovary of the unmated animal.

Uterine Cycle.—So far as the available material goes, it would appear that



FIG. 1.

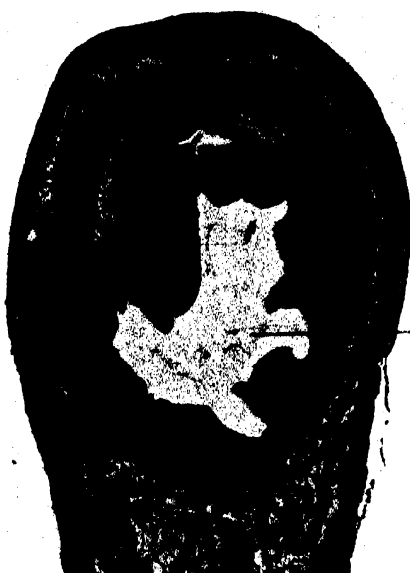


FIG. 3.



FIG. 4.

L.



FIG. 1.



FIG. 3.



FIG. 2.



FIG. 4.

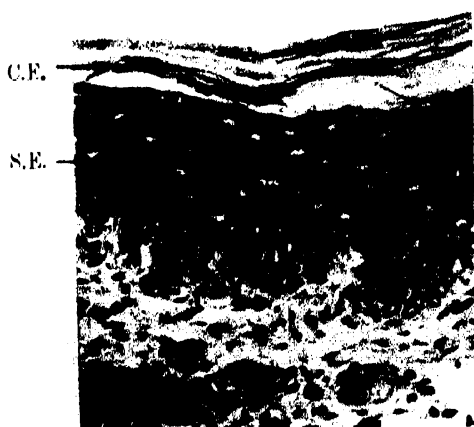


FIG. 5.

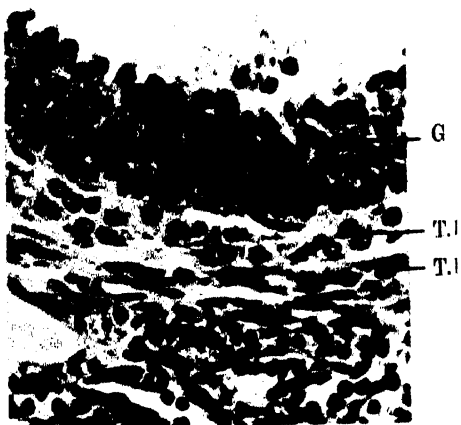


FIG. 6.

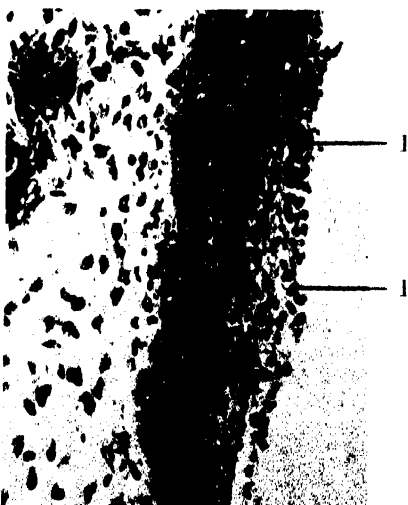
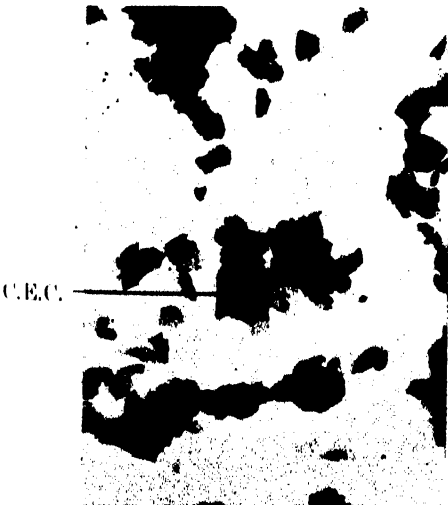


FIG. 3.

FIG. 4.

the extreme condition of uterine dilation found at oestrus in the mouse does not occur in the hamster. The most distended hamster uterus found, taken at a stage when dilation would be complete in the mouse (i.e., second half of oestrus) is shown in Plate 5, fig. 2. While this specimen shows nothing like the condition found in the mouse, it is definitely different from the hamster uteri at other stages of the cycle, which had small discreet lumen. The uterine epithelium shows fairly definite changes during the cycle. In dioestrus the epithelium is low columnar, with the nuclei arranged fairly regularly at the base. Leucocytes are migrating through (Plate 6, fig. 1). The uterine glands appear to be inactive during this stage. At pro-oestrus the epithelium is still low columnar (Plate 6, fig. 2) but leucocytes have ceased to pass through and the glands have become filled with secretion. As the vaginal cornification stage appears, the epithelium becomes taller and the glands begin to empty (Plate 6, fig. 3). In the most distended uterus the epithelium has become 2 to 3 cells thick and large numbers of leucocytes have begun to pass through again (Plate 6, fig. 4).

It is clear that the 4½-day cycle of the unmated hamster is characterised by comparative or complete inactivity of the transitory corpus luteum, and the failure to obtain pregnant or pseudo-pregnant uteri has precluded the study of pre-gestational changes of the endometrium.

VI. Summary.

(1) Specimens of *Cricetulus griseus*, the Chinese hamster, failed to breed under laboratory conditions. This failure, however, could not be traced to any abnormality of the reproductive organs of either sex.

(2) About half the females possessed perfectly normal reproductive organs (the remainder showed various degrees of fatty degeneration of the ovaries), and underwent a succession of short oestrous cycles similar to that of the mouse.

(3) The average length of the cycle was 4·3 days. A summary of the changes occurring in the ovaries, vagina and uterus is given in Table III.

(4) The average number of maturing follicles was eight, so that the size of litter is presumably similar to that of the mouse.

(5) Specimens of *Cricetulus* collected in the field (in the possession of Professor J. P. Hill) show a number of extraordinary bodies in the uterine wall. These bodies are obviously glandular in nature, and would appear to be true myometrial glands (Plate 5, fig. 1). Since they were not found in the laboratory specimens, they would seem to develop during some phase of the fertile cycle.

Table III.—Summary of Events in the Oestrous Cycle of the Unmated Hamster.

Stage of cycle.	Duration.	Vaginal contents.	Vaginal epithelium.	Uterus.	Ovary.
Pro-oestrus	12 hours	Nucleated epithelial cells only.	Proliferation and desquamation of nucleated cells. Stratification setting in.	Lumen not distended. Glands full of secretion. Epithelium low columnar, nuclei at base. No leucocytes migrating through.	Follicles due to ovulate at this oestrus about 0.4 mm. in diameter. Follicles due to ovulate at next ovulation but one not yet distinguishable. Corpora lutea shrinking rapidly.
Oestrus	1½ days	Cornified epithelial cells. Occasionally nucleated cells towards end.	Stratified, with sheets of cornified epithelium sloughing off into lumen.	Lumen becoming distended towards the end. Glands emptying of secretion. Epithelium columnar, nuclei regularly arranged at first, but later becoming irregular with leucocytes migrating through.	Large follicles increasing to about 0.45 mm. in diameter. Corpora lutea down to about 0.25 mm. diameter. Ovulation occurs at the end of this phase.
Di-oestrus	2½ days	Nucleated cells and leucocytes.	4 to 6 cells thick. Leucocytes migrating through in large numbers.	Lumen small. Glands empty. Epithelium low columnar. Nuclei arranged regularly. Leucocytes migrating through.	Corpora lutea form and develop to about 0.55 mm., beginning to shrink towards end. Ova in tube during early part. Follicles due to ovulate at next oestrus increase from about 0.2 mm. to nearly 0.4 mm. in diameter.

The work described was made possible by the kindness of Professor E. Hindle and Professor J. P. Hill, F.R.S., through whom the material was secured. I am also indebted to Miss M. Hill for histological assistance.

The expenses were defrayed from a grant from the Medical Research Council.

DESCRIPTION OF PLATES.

Guide Letters.

C.E., cornified epithelium; *C.E.C.*, cornified epithelial cell; *C.L.*, corpus luteum; *D.L.*, partially distended lumen; *E.*, Epithelium; *G.*, follicular granulosa; *L.*, leucocyte; *M.G.*, myometrial gland; *N.E.C.*, nucleated cell; *S.E.*, stratified epithelium; *T.E.*, theca externa; *T.I.*, theca interna; *U.L.*, uterine lumen; *U.M.*, uterine mucosa.

PLATE 5.

- FIG. 1.—Myometrial gland in wall of hamster uterus (Professor J. P. Hill's material). $\times 25$.
 FIG. 2.—Uterus of Hamster 4 in second half of œstrus, showing partial distention of lumen. $\times 25$.
 FIG. 3.—Vagina of Hamster 4 in œstrus, showing intense cornification of the vagina. $\times 20$.
 FIG. 4.—Corpus luteum in left ovary of Hamster 5, mid diœstrus. Cavity not completely closed. $\times 130$.

PLATE 6.

- FIG. 1.—Uterine epithelium of Hamster 1, mid diœstrus. Low columnar with leucocytes passing through. $\times 450$.
 FIG. 2.—Uterine epithelium of Hamster 5, pro-œstrus. Low columnar, no leucocytes. $\times 450$.
 FIG. 3.—Uterine epithelium of Hamster 8, mid-œstrus. Thickening set in, no leucocytes. $\times 450$.
 FIG. 4.—Uterine epithelium of Hamster 4, late œstrus. Epithelium thickened, with abundance of leucocytes passing through. $\times 450$.
 FIG. 5.—Vaginal epithelium of Hamster 4, cornification. $\times 325$.
 FIG. 6.—Follicle wall in Hamster 8, showing theca interna. $\times 400$.

PLATE 7.

- FIG. 1.—Smear of vaginal contents in diœstrus. Nucleated epithelial cells and leucocytes. $\times 95$.
 FIG. 2.—Smear of vaginal contents in pro-œstrus. Nucleated epithelial cells only. $\times 95$.
 FIG. 3.—Smear of vaginal contents in œstrus. Cornified epithelial cells only. $\times 95$.
 FIG. 4.—Vaginal mucosa in diœstrus. $\times 275$.

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*Heat and Osmotic Change in Muscular Contraction without
Lactic Acid Formation.*

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University College, London.

(Received February 7, 1931.)

It was shown by Lundsgaard (1930, *a*) that a muscle poisoned with mono-iodo-acetic acid (IAA) may give a number of apparently normal contractions without the liberation of any lactic acid. After 50 to 100 twitches a characteristic form of contracture sets in. Phosphagen, as creatine-phosphoric acid (vertebrate muscle), or as arginine-phosphoric acid (crustacean muscle), is broken down (Lundsgaard, 1930, *a*, 1930, *b*, 1931), supplying presumably a large part, if not all, of the energy set free. This paper describes an investigation (*a*) of the total heat liberated during stimulation to complete exhaustion in the absence of oxygen, and (*b*) of the resulting change of osmotic pressure.

A pair of frog's sartorii (Dutch *R. esc.*) was mounted on a "double" thermopile of the usual type (Hill, 1928) and placed in a thermostat maintained within about 0.001° at 20.3° C. They were then soaked, in position, for 2 hours, in phosphate Ringer's fluid (p_{H} 7.2, 10 mg. P/100 c.c.) through which oxygen was bubbled. This was then removed and the procedure described by Hill and Kupalov (1930) was carried out to calibrate the apparatus for vapour pressure change.

(A) The chamber was rapidly washed out with Ringer's fluid of 1.5 times the normal strength (1.5 R).

(B) After half-an-hour the steady deflection $-H_1$ was read, by reversing it in the galvanometer with a copper reversing key.

(C) The chamber was then rapidly washed out with Ringer's fluid of 0.5 times the normal strength (0.5 R).

(D) After half-an-hour the steady deflection $+H_2$ was read as before.

(E) ($H_1 + H_2$) was then taken as the deflection due to a difference of vapour pressure between muscle and chamber equal to that between Ringer's fluid and water.

(F) From this the "increment in resting heat rate in nitrogen" due to subsequent stimulation could be expressed in terms of increased osmotic pressure.

A standard 0.4 per cent. solution of IAA had been prepared from the crystalline material, kindly made for us by Dr. H. R. Ing, and neutralised. This was added to normal phosphate Ringer's fluid, of the same composition as before,

to make a 1/25,000 to 1/12,500 solution. The chamber was filled with this solution and the muscle soaked for 45 minutes; oxygen was bubbled through for the first 30 and pure nitrogen for the last 15 minutes. The solution was then replaced by pure nitrogen, without admitting oxygen, and as soon as the deflection was constant it was read by reversal and stimulation commenced.

Single shocks were given, 30 to 180 per minute, and continued till all sign of heat production had ceased. The total heat was measured from the area of the deflection-time curve (Hill, 1928, p. 120), a suitable resistance being inserted in the circuit to keep the deflection on the scale. The mechanical response was recorded on a smoked drum with an isometric lever. It is not sufficient to continue the stimulation merely so long as the mechanical response is visible. The latter seems to disappear (but see below) long before all the heat has been produced. Stimulation was not stopped until the galvanometer had returned to a constant position for at least a minute; this required 5 to 10 minutes according to the rate. The resistance was then removed and the final deflection was read, as before by reversal, at intervals for several minutes. From the increment in the deflection the vapour pressure change, and thus the increase of osmotic pressure, could be determined. Calibration was finally carried out as usual, and the muscles weighed.

In the earliest experiments it was noticed, as contracture set in, that the mechanical response (as recorded with a lever on a drum) continually diminished, and finally disappeared at a time when there was still obvious heat production. This was thought, at first, to be an example of a phenomenon often looked for but never verified (see Gasser and Hartree, 1924), viz., of "heat production without contraction." Later, when the muscle was connected to a very sensitive optical lever, it was found that a small mechanical response was still to be seen so long as any heat was produced by stimulation. The ratio of tension to heat diminished indeed, but not to zero; presumably the rigidity of the muscles, due to the contracture, diminishes the external manifestation of the mechanical response and the friction of an ordinary lever prevents this diminished response from making any record on smoked paper. Other workers (e.g., Lundsgaard) have found that the phosphagen of an IAA-muscle does not completely disappear on stimulation to apparent exhaustion. Possibly if stimulation had been continued longer they might have found still less phosphagen, or none at all. It is clear in any case that maximal contracture may occur before all the energy is liberated, presumably therefore before all the phosphagen has broken down.

Table I contains a summary of all the valid experiments performed with IAA-muscles. At the end, for comparison, a series of eight experiments (Table II) was made with normal muscles; the mean change of osmotic pressure determined from the latter agrees closely with that given by Hill and Kupalov (1930, p. 467). Each experiment occupied a day, and several

Table I.—Heat and Osmotic Change in Frogs' Muscles (*R. esc.*) Poisoned with Mono-iodo-acetic Acid (IAA) and Stimulated to Exhaustion in Nitrogen.

	Date.					
	Dec. 4.	Dec. 8.	Dec. 9.	Dec. 10.	Dec. 11.	Dec. 12.
Concentration of IAA.....	1 25000	1 25000	1 25000	1 25000	1 20000	1 12500
Minutes soaking.....	45	45	45	60	60	45
Shocks per minute.....	20	—	—	30	30	30
Heat: cal./g.	0.390	0.423	0.452	0.423	0.433	0.478
Osmotic change $\equiv$ p.c. NaCl....	0.222 †	0.151	0.255 †	0.167	0.176	0.157

	Date.					
	Dec. 15.	Dec. 16.	Dec. 17.	Dec. 18.	Dec. 19.	Dec. 20.
Concentration of IAA.....	1 12500	1 12500	1 12500	1 12500	1 12500	1 15000
Minutes soaking.....	60	60	45	60	45	45
Shocks per minute.....	45	45	45	45	45	45
Heat: cal./g.	0.338	0.264	0.295	0.326	0.284	0.303
Osmotic change $\equiv$ p.c. NaCl....	0.160 *	— *	0.114	0.089 *	0.118	0.182

	Date.					
	Dec. 31.	Jan. 1.	Jan. 2.	Jan. 3.	Jan. 4.	Jan. 5.
Concentration of IAA.....	1 25000	1 20800	1 17800	1 15800	1 13900	1 12500
Minutes soaking.....	45	45	45	45	45	45
Shocks per minute.....	45	45	45	45	45	45
Heat: cal./g.	0.258	0.375	0.355	0.364	0.293	0.391
Osmotic change $\equiv$ p.c. NaCl....	0.112	0.156	0.137	0.156	0.107	0.137

	Date.						
	Jan. 6.	Jan. 7.	Jan. 17.	Jan. 19.	Jan. 21.	Jan. 23.	Mean.
Concentration of IAA	1 12500	1 15800	1 14000	1 14000	1 15800	1 15800	
Minutes soaking.....	45	45	45	45	45	45	
Shocks per minute.....	180	180	45	45	45	45	
Heat: cal./g.	0.461	0.380	0.352	0.325	0.334	0.316	0.367
Osmotic change $\equiv$ p.c. NaCl....	0.159	0.156	0.077 †	0.078 †	0.137	0.136	0.147

* Omit; too long soaking at high concentration.

† If these two high and two low values of the osmotic change be omitted the mean value is practically unchanged.

Table II.—Heat and Osmotic Change in Normal Frogs' Muscles (*R. esc.*) Stimulated to Exhaustion in Nitrogen.

	Date.								Mean.
	Jan. 12.	Jan. 13.	Jan. 14.	Jan. 15.	Jan. 16.	Jan. 18.	Jan. 20.	Jan. 22.	
Shocks per minute....	94	90	45	45	45	45	45	45	—
Heat: cal./g.	0.906	0.712	0.623	0.934	0.754	0.871	0.772	0.554	0.766
Osmotic change $\equiv$ p.c. NaCl	0.243	0.194	0.246	0.296	0.269	0.276	0.268	0.210	0.250

Reckoned per cal./g. the mean osmotic change is 0.326 per cent. NaCl. The mean value of the same quantity in the experiments of Hill and Kupalov (1930) is 0.335.

rather delicate measurements had to be made, so that (taking account of the variability of the material) too great a consistency need not be looked for. The quantities given in the tables are (A) the total heat in stimulation to exhaustion in nitrogen, reckoned in calories per gramme, and (B) the osmotic pressure change expressed in terms of the percentage of NaCl which would need to be added to Ringer's fluid to produce the same effect. It is seen that in the IAA-muscles—

(A) the total heat to exhaustion is 0.367 cal./g.;

(B) the osmotic change is equivalent to 0.147 per cent. of NaCl added to Ringer's fluid.

We will consider (B) first. In four experiments Meyerhof (1930) found, in IAA-muscles, a difference of freezing point, before and after "complete fatigue," of 0.053°, 0.0435°, 0.036°, 0.045°—mean 0.0445°. This would correspond to an added 0.076 per cent. NaCl only. It is impossible to reconcile this with the present experiments, for not one of our readings, out of 21, is so low. Possibly there was some consistent error in the freezing point measurements; possibly the muscles were not stimulated to so complete exhaustion as those in the present experiments (there was still on the average 7 mg. P/100 g. of phosphagen left), possibly spontaneous breakdown had begun before the beginning of stimulation—the average initial phosphagen content in Meyerhof's four muscles was only 53 mg. P/100 g. This spontaneous breakdown is liable to occur whenever muscles are exposed to IAA at too high a concentration, or for too long a time. In Table I the experiments of December 15, 16 and 18 are omitted from the mean for this reason. In the other experiments there is no reason to suppose that the spontaneous

breakdown had begun. Let us see how far our observed osmotic change can be explained.

So far as present knowledge goes, the chemical processes accompanying contraction in a frog's muscle adequately poisoned with iodoacetic acid are (Lundsgaard, 1930, *b*):—

- (a) The breakdown of creatine-phosphoric acid to creatine and phosphate;
- (b) The breakdown of adenylyl-pyrophosphoric acid to inosinic acid and two molecules of orthophosphate, together with the formation of one molecule of ammonia;
- (c) The esterification of the newly formed phosphate with hexose (probably derived from glycogen), some 30 per cent. of the P being combined as the mono- and 70 per cent. as the di-phosphoric ester.

If x millimoles per 100 g. of muscle be the concentration of phosphagen in the resting poisoned muscle, and y millimoles per 100 g. the concentration of adenylyl-pyrophosphoric acid, and if we suppose *the whole* of these to be broken down in stimulating to exhaustion, the final concentrations will be, in millimoles per 100 g. :—

Creatine x : hexose monophosphoric ester $0.3(x + 2y)$: hexose diphosphoric ester $0.35(x + 2y)$: ammonia y : inosinic acid y ; total $(1.65x + 3.3y)$.
The increase therefore is $(1.65x + 3.3y) - (x + y)$, i.e., $(0.65x + 2.3y)$.

Now the average concentration of phosphagen in a resting muscle poisoned with iodoacetic acid, according to 33 determinations given in Lundsgaard's paper (1930, *b*), is equivalent to 61 mg. P, or 1.97 millimoles, per 100 g. of muscle. The average deviation from the mean is only 7 per cent. The average concentration of pyrophosphate in a poisoned muscle, according to six observations of Lundsgaard (1930, *b*) and two of Meyerhof (1930), is equivalent to $20\frac{1}{2}$ mg. P, or 0.33 millimole, per 100 g. Putting $x = 1.97$ and $y = 0.33$ in the above formula, the maximal increase of concentration (assuming the whole of each substance to be broken down) is 2.04 millimoles per 100 g.*

Let us assume the "free" water of muscle to be 0.77 g. per gramme of muscle (Hill, 1930), and these new molecules to be dissolved in this water. Let us further assume that they cause a normal depression of vapour pressure, viz., that 1 millimole per 100 g. H_2O produces a relative depression $(p_0 - p)/p_0$.

* We have neglected the ionic changes associated with the production of new molecules. The formation of anions, or the disappearance of cations, causes unionisation of protein or other buffer substances; the formation of cations, or the disappearance of anions, results in their ionisation; the total K^+ and Na^+ free in solution remains unaltered. See Hill and Kupalov (1930, p. 472).

of 0.000180. The relative depression, therefore, due to 2.04 millimoles per 100 g. of muscle will be $2.04 \times 0.000180 / 0.77 = 0.000477$. Now in the region of 0.7 per cent. NaCl the relative depression for 1 millimole NaCl per 100 g. H_2O is 0.000330, so that the calculated depression due to the new molecules in muscle is equivalent to that of $0.000477 / 0.000330 = 1.44$ millimole = 0.085 g. of NaCl in 100 g. H_2O . The average observed depression is equivalent to that of 0.147 g. of NaCl in 100 g. H_2O . *The calculated change therefore is rather more than half that actually observed.*

It is not easy to see, if we assume that phosphagen and adenylyl-pyrophosphoric acid actually pre-exist in muscle as separate molecules, and without introducing other chemical reactions into the picture, how the calculated amount can be increased, for—

- (1) we have assumed the whole of the phosphagen and the whole of the pyrophosphate to be broken down ;
- (2) we have assumed none of the pre-existing inorganic phosphate to be removed by esterification ;
- (3) we have taken the full normal value for the molal depression of vapour pressure of the substances formed, and have imagined them dissolved in rather less than the total water ;
- (4) the change of vapour pressure was measured 10 minutes at least after the end of stimulation, so allowing plenty of time for the esterification to occur ;
- (5) we have assumed the whole of the hexose to be derived from glycogen and so to constitute new, and not pre-existing, molecules : according to information received from Dr. Lundsgaard the amount of glycogen broken down is not more than the amount of hexose esterified.

Even if we assumed no esterification, or (what is the same thing for the calculation of the number of molecules produced) that only the hexose monophosphoric ester was formed, the increase calculated would be $(x + 3y) = 2.96$ millimoles per 100 gramme of muscle, instead of 2.04, which would still give only 84 per cent. of the observed depression of vapour pressure.

One possibility, however, remains, viz., that the phosphagen and the adenylyl-pyrophosphoric acid do not occur in the molecular form in resting muscle, but are held as part of a larger complex, so not affecting the osmotic pressure. In that case the calculated increase of concentration due to exhaustion would be $(1.65x + 3.3y)$ which, with $x = 1.97$ and $y = 0.33$, is 4.24 millimoles per 100 g. of muscle, instead of 2.04. The equivalent increase of NaCl concentration would be 0.176 per cent., actually somewhat larger than the observed

amount. There is no difficulty, therefore, in explaining the observed increase of osmotic pressure if we assume phosphagen and pyrophosphate in the resting muscle to be held in some combination and so not to affect the initial osmotic pressure. There is some evidence, indeed, for this (Eggleton, 1930) in the fact that neither diffuses out of the resting muscle, although phosphate, creatine and ammonia do, and creatine-phosphoric acid diffuses through a parchment membrane. Otherwise we cannot explain the observed depression of vapour pressure in exhaustion, without assuming some rather extensive chemical reaction hitherto unknown.

With regard to the total heat, Meyerhof, Lundsgaard and Blaschko (1930) in a preliminary note state that they found, in seven experiments on IAA-muscles, an average of 146 cal. per 1 g. of H_3PO_4 broken off from phosphagen. In their paper to be published shortly (private communication) the four most significant experiments gave 148, 159.5, 175 and 144, mean 157 cal. per 1 g. H_3PO_4 . This is just 500 cal. per 1 g. of phosphagen P set free. In our experiments, with this value, 0.367 cal. per 1 g. muscle would require 0.73 mg. of phosphagen P per 1 g. of muscle to be liberated. This is not an impossible amount, but rather high, though it agrees with Lundsgaard's estimate.* Assuming, as above, a value of 0.61 mg., the heat per 1 g. of phosphagen P would be 600 instead of 500 cal. This is scarcely outside the range of Meyerhof's results. In view of the difference in material (30 to 50 g. of muscle poisoned by injection in the one case, 150 mg. poisoned by soaking in the other, the former fresh, the latter after soaking for some hours in Ringer's fluid) and of the difference in technique (calorimetric as compared with myothermic), the agreement is very satisfactory. We may assume a value of 500 cal. per 1 g. of phosphagen P set free.

According to Meyerhof and Lohmann (1928) the heat of hydrolysis of creatine-phosphoric acid in neutral solution is 10,000 to 11,000 cal. per gramme molecule, say, 340 cal. per 1 g. P. The value observed in the intact but poisoned muscle being 500 cal., there is clearly 160 cal. missing. As a matter of fact, 500 cal. per 1 g. P is exactly the value found by Meyerhof and Suranyi (1927) for enzymatic hydrolysis in neutral solution, Meyerhof, Lundsgaard and Blaschko, however, appear to regard the value for acid hydrolysis as more

* Dr. E. Lundsgaard, whom we have consulted, believes that an initial phosphagen content equivalent to 75 mg. P/100 g. is not impossible for the muscles employed-Sartorii, he states, regularly have rather more phosphagen than *gastrocnemii*. This would raise the calculated value of the osmotic pressure change from 0.085 to 0.087 per cent. NaCl. This is still well below the observed value (0.147 per cent.) so some other factor is still required.

accurate. The esterification of glycogen with phosphate might yield a small contribution (Meyerhof and Suranyi, 1927); so might the splitting of adenylypyrophosphoric acid; the thermal effects of slight changes of p_H are presumably involved; possibly, as suggested above on the evidence of the osmotic changes, either (a) some other reaction, hitherto unknown and involving appreciable energy, occurs during activity, or (b) the creatine-phosphoric acid and the adenylypyrophosphoric acid do not exist as such in resting muscle, but each as part of a more complex molecule containing more energy. Lastly, a slight formation of lactic acid (or methyl glyoxal) may have occurred, in spite of the poisoning which was certainly very effective. Even 0.025 per cent. of lactic acid formed would raise the heat by as much as 0.075 cal. per 1 g. It is not possible at present to decide between these various alternatives.

One point remains to be considered, the caloric quotient of lactic acid. In a normal muscle stimulated to extreme fatigue in nitrogen (autumn frog) about 1 cal. per gramme of heat is set free and about 0.3 per cent. of lactic acid liberated. The heat, per 1 g. of lactic acid, is about 350 cal. (see Hill and Kupalov, 1930, p. 460, quoted from Meyerhof), so that 1 cal. $\equiv$ 2.85 mg. of lactic acid. If now we may assume that, in stimulation to extreme fatigue, the same energy-producing reactions occur in the normal as in the IAA-muscle (in addition to lactic acid formation in the former), we must subtract from 1 cal. the amount of heat found above, viz., 0.367 cal., leaving 0.633 cal. as the heat associated with the formation of the 2.85 mg. of lactic acid.

The heat of transformation of glycogen to lactic acid, according to Meyerhof and Suranyi (1927), is 176 cal. per gramme, so that 2.85 mg. corresponds to 0.502 cal. To this must be added the heat of neutralisation of the acid. If no phosphagen change occurred we could assume (see Stella, 1929) a value for the latter of 9400 cal. per gramme molecule or 104 cal. per gramme. In the breakdown, however, of 1 g. mol. of phosphagen at p_H 7.2 (resting muscle) to 1 g. mol. each of creatine and phosphate at p_H 6.5 (fatigued muscle), according to the curves of Meyerhof and Lohmann (1928, p. 66), there is a freeing of about 0.65 equivalent of alkali; phosphagen split to the extent of 0.6 mg. P per 1 g. muscle, between these p_H limits, would yield therefore 0.0126 milli-equivalents of alkali, which would neutralise 1.13 mg. of lactic acid. The heat of this neutralisation would be small. The remaining 1.72 mg of lactic acid would yield by its neutralisation 0.179 cal. so that the calculated total heat in the formation and neutralisation of lactic acid would be $0.179 + 0.502 = 0.681$ cal. per 1 g. muscle, which is not far

from the value, 0.633 cal., deduced above. We may conclude, therefore, that if the energy-liberating reactions occurring in the IAA-muscle occur also in the normal one, they, together with lactic acid formation and neutralisation, are adequate to account for the total energy set free in stimulation of the normal muscle anaerobically to complete fatigue.

Summary.

(1) In frogs' sartorii (Dutch *R. esc.*, December and January) poisoned by soaking with mono-iodo-acetic acid, in which presumably (Lundsgaard) no lactic acid was formed, stimulation to complete exhaustion in the absence of oxygen yielded, on the average, 0.367 calorie per 1 gramme of muscle, and led to an increase of osmotic pressure equivalent to the addition of 0.147 per cent. of NaCl to Ringer's fluid.

(2) This change of osmotic pressure cannot be explained without assuming, either (a) that some chemical reaction, hitherto unrecognised, occurs in stimulated poisoned muscles, or (b) that phosphagen and adenylyl-pyrophosphoric acid exist, not as simple molecules in the resting muscle, but bound in some more complex form from which they are broken off during activity.

(3) The total heat is in good agreement with recent observations by Meyerhof, Lundsgaard and Blaschko. In the poisoned muscle about 500 calories are liberated per 1 g. of phosphagen phosphorus set free. The source of this heat is discussed.

(4) In the sartorius of the normal autumn frog about 1 calorie per 1 g. of muscle is set free during stimulation, in the absence of oxygen, to exhaustion. If the energy liberating reactions of the poisoned muscle occur also in the normal one, they, together with lactic acid formation and neutralisation, are sufficient to account for the whole of the heat produced.

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A Spectrographic Analysis of Animal Tissues.

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[PLATE 8.]

1. Introduction.

It is recognised that, in addition to those chemical elements which are known to be necessary and essential constituents of protoplasm, others have a wide distribution in living matter. Such are iron, copper, zinc, aluminium and manganese. Besides these, a number of additional elements have been recorded as occurring spasmodically in animal and plant tissues. Hitherto the spectroscope has rarely been used as a method of detecting uncommon elements in animal tissues; yet the spectroscope supplies a valuable tool for making a wide survey of tissue contents with a rapidity of which chemical methods do not allow. The present paper is the first report on such a survey, carried out on a quantitative basis. The field to be covered is, of course, vast. As a beginning, we have restricted ourselves mainly to annelids, in which whole animals were analysed, and molluscs, the separate organs of which were dissected out and dealt with. In addition, a limited amount of other material has been studied, including human organs, insects, and a few representatives of other groups. As the work progresses it is intended to investigate the principal divisions of the animal kingdom.

2. Methods and Material.

The spectrographic method used has already been outlined by one of us (Ramage, 1929). When a whole animal, such as a worm, is to be analysed, the living animal is first washed thoroughly under the tap, and then in distilled water. The animal is next placed in a crucible, minced with scissors, and dried at 100° C. If separate tissues are to be studied, the animal is killed without a fixative, the tissues are dissected out, and then treated in the same way as before. Needless to say, every possible precaution is taken during the operations to avoid contamination. The dried specimen is powdered in an

agate mortar and a quantity of 50 mg. of the dried tissue is then weighed and rolled in an ashless filter paper. If a liquid such as blood is to be analysed, 0.1 c.c. is pipetted on to a rolled ashless filter paper. The roll is burnt completely in an oxy-coal gas flame in front of the slit of a quartz spectrograph, using a quartz lens to focus the image of the flame on the slit, and the spectrum is photographed.

The elements which can be detected by this method are the following : *lithium, sodium, potassium, rubidium, caesium, copper, silver, calcium, strontium, barium, magnesium, cadmium, gallium, indium, thallium, lead, phosphorus, bismuth, chromium, manganese, iron, cobalt, nickel, ruthenium, rhodium, palladium, iridium*, and in addition, *calcium fluoride*. We have found the substances in italics in all or some of the tissues analysed. It will be observed that there are metals known to occur in animals which are not included in the list. They are : aluminium, arsenic, boron (Bertrand and Agulhon, 1913), antimony (Chapman, 1930), bismuth (Fairley and Burrell, 1918), gold (Berg, 1928), molybdenum (Mankin, 1928), tin (Misk, 1923), titanium (Baskerville, 1899; Azéma and Pied, 1930), uranium (Bishop, 1928), vanadium (Henze, 1913; Phillips, 1918), and zinc. The reason why these elements cannot be detected by our method is that the thermal or electrical conditions of the flame are not sufficiently intense either (a) to reduce the oxides in adequate quantities, or (b) when small quantities only are present, to excite the vibrations producing the lines with enough energy to photograph them, placed, as most of them are, in the further portion of the ultra-violet region of the spectrum, i.e., beyond wave-length 3100.

The method was used quantitatively in the following manner. A standard solution was prepared, containing the various elements in known amounts. On each plate the spectra of four different measured volumes of this solution were photographed, together with the spectra of the tissues to be analysed (see Plate 8). The intensity of a given line in a tissue spectrum could then be matched with the corresponding line in one of the standard spectra and the amount of the element in the tissue deduced. Quantities of elements present in tissues are given below in percentages of tissue dry weight.* Whenever the statement is made that an element is absent in a given specimen, absence in quantity sufficient to be detected by our method is, of course, meant.

Table I gives a list of the specimens from which spectrograms were made. Generic and specific names of animals are given in the list; for brevity, genera

* The dry weight of *Terebella lapidaria*, taken as an example, must be $\times 6.7$ to convert it to wet weight.

only will be mentioned in the text. When, in the text, an element is stated to be present in a certain organ of an animal, or in a certain animal out of a group, it is implied that the element in question is absent from the remaining organs of the animal, or animals in the group, which are named in Table I.

We wish to thank the following lady and gentlemen for kindly supplying material: Miss M. V. Lebour, Dr. P. A. Buxton, Professor H. Graham Cannon. Dr. A. J. Grove, Messrs. R. Hancock, A. G. Lowndes, H. G. Newth, Professor J. H. Orton, Dr. F. S. Russell, Mr. D. P. Wilson, and Professor Haswell Wilson. We desire also to acknowledge a grant from the Government Grant Committee of the Royal Society which defrayed the cost of some of the apparatus.

Table I.—List of specimens of which spectra have been made.

Group.	Animal.	Individuals.	Locality.
A.—Whole Animals Studied.			
Porifera	<i>Clathrina coriacea</i>	2	Plymouth, Roscoff.
Cœlenterata	<i>Cosmetira pilosella</i>	Numerous	Plymouth.
	<i>Beroë</i> sp.	"	"
Nemertea	<i>Carinella</i> sp.	1	"
Chaetognatha	<i>Sagitta bipunctata</i>	Numerous	"
Gephyrea	<i>Thalassema neptuni</i>	Several	"
	<i>Phascolosoma pellucidum</i>	2	Salcombe.
	<i>Sipunculus nudus</i> , body wall	1	Roscoff.
Annelida	<i>Sthenelais boa</i>	Several	Plymouth, Millport.
	<i>Nereis cultrifera</i>	"	Plymouth.
	<i>Nephtys</i> sp.	"	"
	<i>Owenia fusiformis</i>	"	Torquay.
	<i>Audouinia tentaculata</i>	3	Plymouth.
	<i>Amphitrite edwardsii</i>	1	Salcombe.
	<i>Terebella lapidaria</i>	Several	Plymouth.
	<i>Lemice conchigela</i>	"	"
	<i>Melinna palmata</i>	"	"
	<i>Notomastus latericius</i>	"	Salcombe.
	<i>Arenicola marina</i>	2	Plymouth.
	<i>Diopira voluticornis</i>	1	"
	<i>Branchioma variolosum</i>	Several	"
	<i>Spirographis spallanzanii</i>	3	Roscoff.
	<i>Sabella pavonina</i>	2	Salcombe.
	<i>Myxicola infundibulum</i>	5	Salcombe, Plymouth, Roscoff.
	<i>Sabellaria alveolata</i>	Several	Roscoff, Plymouth, Widemouth Bay.
	<i>Hirudo medicinalis</i>	1	—
	<i>Lumbricus terrestris</i> *	13	Cambridge, Liverpool, London, Marlborough, Sheffield.
Crustacea	<i>Calanus finmarchicus</i>	Numerous	Plymouth.
Myriapoda	<i>A centipede</i>	Several	Birmingham.
Arachnida	<i>Epeira diademata</i>	"	"
	<i>E. umbratica</i>	"	"
	<i>Oronothodorus moubate</i>	Numerous	—

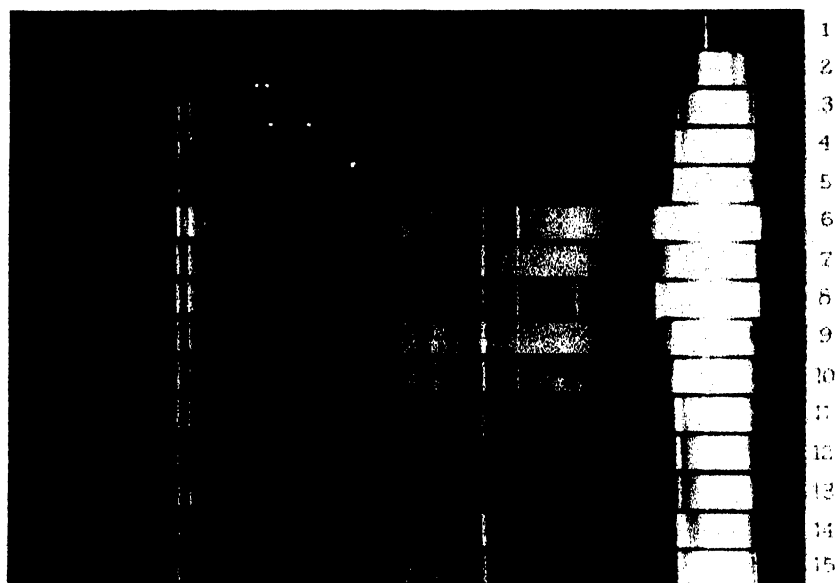
* The worms were first fed with ashless filter paper until no earth came out in the faeces.

Table I—(continued).

Group.	Animal.	Individuals.	Locality
A.—Whole Animals Studied—(continued).			
Insecta	<i>Musca domestica</i> , larvæ	Numerous	Birmingham.
	<i>Chironomus</i> sp., larvæ	"	"
	<i>Dytiscus</i> sp., larvæ	Several	Newdigate.
	" adult	1	"
	<i>Stauroderus bicolor</i>	Several	Birmingham.
	<i>Pieris brassicae</i> , larvæ	"	Suffolk.
	<i>Agrotis ripæ</i> , larvæ	"	"
	<i>Vanessa urticae</i> , larvæ	"	"
	<i>Chærocampa elpenor</i> , body wall of larvæ.	"	"
Gastropoda	<i>C. porcellus</i> , body wall of larvæ	"	"
	<i>Limacina retroverea</i>	Numerous	Plymouth.
B.—Tissues Studied separately.			
Echinodermata	<i>Paracentrotus lividus</i>	2	Roscoff.
	<i>Echinus esculentus</i>	1	Plymouth.
Annelida	<i>Aphrodite aculeata</i>	Several	Plymouth, Millport.
Gastropoda	<i>Patella vulgata</i>	"	Plymouth.
	<i>Haliotis tuberculata</i>	"	Roscoff.
	<i>Aplysia punctata</i>	"	Salcombe.
	<i>Archidoria tuberculata</i>	"	Plymouth.
	<i>Aeolidia papillosa</i>	"	Millport.
	<i>Helix aspersa</i>	"	Cambridge, Liverpool, London, Marlborough, Plymouth.
	<i>Helix pomatia</i>	"	Newdigate.
	<i>Arion ater</i>	"	Birmingham.
	<i>Pecten maximus</i>	11	Plymouth, Millport.
Lamellibranchiata	<i>Pinna pectinata</i>	1	Salcombe.
	<i>Ostrea edulis</i>	2	Plymouth.
	<i>Anodonta cygnea</i>	Several	Liverpool, Newdigate, Sheffield.
			Plymouth.
Cephalopoda	<i>Sepia officinalis</i>	1	Plymouth.
	<i>Loligo forbesii</i>	2	"
Vertebrata	Man	3	Norwich, Birmingham.
C.—Blood only.			
Crustacea	<i>Astacus fluviatilis</i>	Several	Newdigate.
	<i>Maia squinado</i>	"	Plymouth.
	<i>Carcinus maenas</i>	"	"

3. Iron and Copper.

Our results indicate that iron and copper are both essential constituents of protoplasm. One hundred and forty-six different spectrograms have been made, either from whole animals or from living tissues, that is to say tissues other than skeletal matters, all of which show the lines both of iron and of



TYPICAL EMISSION SPECTRA OF ANIMAL TISSUES.

No. 1 is the spectrum of the filter paper used throughout. The red end of each spectrum is on the right. Nos. 2, 3, 4 and 15 are the spectra of four different quantities of a standard solution, namely, 0.02 c.c., 0.05 c.c., 0.1 c.c., and 0.2 c.c. respectively. This standard solution had the following percentage composition: Na, 0.15; K, 0.2; Ca, 0.02; Mg, 0.05; Fe, 0.056; Cu, 0.005; Ag, 0.0025; Mn, Ni, Li, Rb, Sr, each 0.002; P, 0.6. The intensities of the lines in the spectra of the animal specimens can be matched with corresponding lines on one of the standard spectra and the quantities of the elements in question thus determined.

The principal lines of the more important elements are indicated on the plate by white ink spots. They are as follows: On spectrum No. 2, Cu; No. 3, Ag; No. 4, Ni; No. 5, Pb; No. 6, Sr; No. 8, calcium fluoride band; No. 11, Mn; No. 12, Li; No. 15, Rb.

All of the spectra show Fe and Cu, in addition to Na, K, Ca and Mg. Below are given the most important additional characters of each spectrum.

No. 5 (specimen 80): medusa, *Cosmetira pilosella*, Pb (strong), Mn, Li, Sr.

No. 6 (specimen 480): sponge, *Clathrina coriacea*, Sr (strong), Mn.

No. 7 (specimen 91): polychæte worm, *Notomastus latericius*, Mn (a trace), Li, Rb, Sr.

No. 8 (specimen 373): body-wall of a nudibranch mollusc, *Archidoris tuberculata*, CaF_2 , Sr (very strong).

No. 9 (specimen 406): polychæte worm, *Myxicola infundibulum*, Mn, Ni, Co, Ag, Li, Sr.

No. 10 (specimen 362): liver of scallop, *Pecten maximus*, Cd, Mn, Ag (strong), Li, Sr.

No. 11 (specimen 304): liver of snail, *Helix aspersa* from Marlborough, Mn (strong), Pb, Li, Sr.

No. 12 (specimen 193): liver of *Helix aspersa* from Plymouth, Mn (strong), Ag, Li, Rb. Contrast No. 11 with Pb and Sr, No. 12 with Ag and Rb.

No. 13 (specimen 185): common genital duct of snail, *Helix pomatia*, Mn, Sr (strong).

No. 14 (specimen 10): common genital duct of slug, *Arion ater*, Mn (very strong), Li. Contrast Sr and Mn in Nos. 13 and 14.

copper (see Plate 8). Of these 146 specimens, 45 were prepared from whole animals. Twenty-four species of annelids, 1 sponge, 1 medusa, 1 ctenophore, 1 nemertean, *Sagitta*, 1 mollusc, 1 copepod, 1 centipede, 3 arachnids, and 10 insects were studied in this manner. It is, of course, possible that in these cases compounds of iron and copper may have been adhering to the outsides of the animals, they may have been in the skeletal structures, or they may have been in the gut contents. But this objection does not hold with the remaining 101 specimens. These were prepared from dissected organs of the various animals, namely, 14 species of molluscs, 2 echinoids, and human tissues. It can be objected that in the case of the molluscs the copper was furnished by hæmocyanin. This was undoubtedly so in a number of instances, but nevertheless the unfailing presence of both copper and iron in all spectra points to these two elements always being present in protoplasm.

Our conception, then, of the chemical composition of protoplasm must be extended. Not only are the elements C, H, O, N, P, S, Na, K, Mg, Ca and Cl necessary to its make-up, but iron and copper appear to be essential as well. The wide distribution of iron in living matter is already well known (Warburg, 1921, and others), and the recent work of Keilin (1925, 1926, 1929) on hæmatin and cytochrome leads one to expect an almost universal distribution of this element in protoplasm. But the presence of copper in all tissues is more surprising, although the presence of copper in animal tissues has previously been recorded by various investigators. Maquenne and Demoussay (1920) found copper to be universal in plants. Most was found in young active tissues; buds contained more than wood or bark. The only known part played by copper in metabolism, apart from its occurrence in hæmocyanin, is its connection with hæmoglobin synthesis. Rats can be made anæmic on a milk diet. When subsequently iron is supplied in their food, the animals cannot utilise this element to build up hæmoglobin unless copper is also present (Waddell, Steenbock, Elvehjem and Hart, 1928). Elvehjem (1931) has also shown that copper aids the synthesis of cytochrome *a*.

It will be shown below that there are other metals which have a wide distribution in protoplasm, without their apparently being present in all tissues.

Copper is present in sea-water. Rose and Bodansky (1920) found 0.14 mg. per kilogram in inshore water at Galveston.

4. *Manganese.*

The wide distribution of manganese in animals and plants is already well known. Bertrand and Rosenblatt (1921) showed its apparently universal presence in plants. In animals, Bertrand and Medigreceanu (1913) analysed the amounts of manganese in certain invertebrates and in organs of vertebrates, Reiman and Minot (1920) in human tissues, Phillips (1917) in certain marine invertebrates, and Wang-tai-si (1928) in marine molluscs. Manganese has been detected in sea-water (Clarke, 1924).

Our spectrograms show a very wide distribution of manganese (see Plate 8). It occurred in all of the 19 species of polychaetes. It was present in all the organs of the land gastropods, being absent only in shells (see Table II). The table shows a considerable constancy in manganese content of the organs of *H. aspersa* from different localities. It shows also that there are the same relative concentrations in the different organs of *H. aspersa* and of *H. pomatia*. The high concentration in the common genital duct of *Arion* (Plate 8, No. 14) however, is remarkable.

Table II.—Distribution of Manganese in Land Gastropods.

7, more than 0.008 per cent. ; 6, 0.008 per cent. ; 5, 0.004 per cent. ; 4, 0.002 per cent. ; 3, 0.0008 per cent. ; 2, less than 0.0008 per cent. ; 1, a trace ; 0, no trace.

Each entry refers to a separate specimen.

	Foot.	Liver.	Kidney.	Common genital duct.	Albumen gland.	Dart sac.	Shell.
<i>H. aspersa</i> —							
Cambridge	3	6, 6, 5	3	2	2	1	0
Liverpool	3	6, 6	3, 2	2	3	1	0
London	3	6, 6	1	1	2	1	0
Mariborough	3	6, 6	3	2	3	1	0
Plymouth	2	6, 4, 6	2	2	3	1	0
<i>H. pomatia</i> *	3, 2, 2 Body wall	7, 7	1, 1	2, 2	2	2, 2	0, 0
<i>Arion</i>	6	5	—	7	2	—	—

* Manganese present also in salivary gland, crop, penis, mucous gland.

Table III.—Distribution of Manganese in Lamellibranchs.

The numbers have the same significance as in Table II. In addition, "8" denotes an unusually high concentration of Mn.

	Foot.	Mantle.	Gills.	Ant. adductor.	Post. adductor.	Liver.	Kidney.	Testis.	Ovary.	Gonad.	Shell.
<i>Anodonta</i> —											
Liverpool	3, 2	7, 6	8	—	—	5	7	—	7, 7	—	2
Newdigate	3, 4	7, 7	8	—	—	8	8	—	7	7	6
Sheffield	7, 7	7, 7	8, 8	—	—	8, 8	8	—	8	8	5
<i>Pecten</i> —											
Eddystone	—	0	0	0	0	2	7	5	2	—	—
Mewstone	—	0	0	1	0	2, 2	7	3	1	—	—
Millport	—	0, 0	1, 1	2	0	2, 2	7	3	2	—	—
<i>Ostrea</i>	—	4	4	0, 0	0, 0	1	—	—	—	1, 2	—
<i>Pinna</i>	0	0	0	—	—	1	3	—	2	—	—

Among the marine gastropods, manganese was found in all the organs of *Æolidia* and *Archidoris*, but there was a noticeable absence of the element from all organs of *Haliotis*.

In the lamellibranchs, *Anodonta* proved to be very rich in manganese. Table III shows this, and also the fact that the concentration varied with locality, particularly in foot and liver. The table shows too that the marine forms were poorer in manganese than the fresh-water forms.

Bradley (1907, 1910) analysed the manganese content of *Anodonta* and *Unio* from various parts of the United States. He maintains that the manganese content of tissues varies considerably with locality and increases with size of individual. As he did not identify his species one cannot be sure that his material was uniform. Bradley found, however, that the fresh-water mussels fed on a manganese-containing plant, *Crenothrix*. In lakes where this organism is absent there are no mussels. Boycott (1917, 1921) analysed the manganese content of 79 species of English land and fresh-water molluscs. Considerable variation in manganese content with locality was found.

There is evidence that manganese plays a functional rôle in certain plants and animals, and that in others, without its necessarily having a function, it manifests effects. The evidence is as follows :—

(1) Bertrand (1897) showed that laccase, the enzyme responsible for the oxidation of latex to lacquer, contains manganese, and that the oxidising power of laccase is proportional to its manganese content.

(2) There is most manganese in those parts of plants with a most active metabolism (Bertrand and Rosenblatt, 1921, 1922).

(3) Manganese appears to be essential for the development of chlorophyll (McHargue, 1919, 1924, 1926, *a* ; Bishop, 1928).

(4) Manganese is concerned in the sporulation of *Aspergillus niger* (Bertrand, 1912).

(5) The growth of rodents is stimulated by manganese (McHargue, 1926, *b* ; McCarrison, 1927 ; Bertrand and Nakamura, 1928).

(6) Titus and Hughes (1929) maintain that manganese, like copper, is necessary for the synthesis of hæmoglobin by anæmic rats when subsequently they receive iron. Waddell and co-workers (1929) say that copper alone, not manganese, is effective.

(7) Harrison and Garrett (1926) and Harrison (1928) induced melanism in Lepidoptera by feeding larvæ with manganese salts. If manganese were generally involved in the production of melanin one might expect to find high proportions of this element in melanic tissues. We found, however, no manganese in the ink sac (wall plus contents) of *Sepia*, although manganese was present in liver and pancreas. In the black body-wall of *Arion* it is true that the manganese content was high, but it was higher still in the colourless genital duct (see Table II).

Finally, it may be observed that the fact of the manganese content of an organ being variable within considerable limits, as for instance that of the liver of *Anodonta* (see Table III), does not preclude a function. A superfluous excess of the element may accumulate when it is available.

5. Nickel and Cobalt.

The existence of both nickel and cobalt in animals is known through the work of Bertrand and Mâchebœuf (1925). They demonstrated nickel and cobalt in organs of man, other mammals, birds and fishes, and in the silk-worm, a crustacean, molluscs, and a tunicate. In the invertebrates, separate organs were not studied. Plants also contain nickel and cobalt (Bertrand and Mokragatz, 1922).

We have found that nickel and cobalt show a spasmodic distribution. The lines of nickel are more frequently present than those of cobalt.

Nickel occurred in 5 out of the 19 species of polychætes and in 7 out of 15 molluscs. The polychætes containing nickel were the following: (1) *Aphrodite*, 0.002 per cent. in the body wall of one from Millport, none in another individual from Plymouth; (2) *Audouinia*, 0.0014 per cent. in one, a trace in

two other individuals; (3) *Arenicola*, 0·0008 per cent. in one, a trace in another specimen; (4) *Spirographis*, 0·0008 per cent. in two individuals, none in a third; (5) *Myxicola*, 0·008 to 0·01 per cent. in each of five specimens (Plate 8, No. 9). It is clear that certain forms, such as *Myxicola*, have a predilection for nickel whatever locality they come from (see Table I for localities), but that in other species some individuals may be with, others without, this element, even when they are of the same provenance.

Among the molluscs, nickel was present only in the following: *Haliotis* (0·004 per cent. in the foot), *Aplysia* (0·004 per cent. in the liver and a trace in the crop), and *Pinna* (0·004 per cent. in the kidney and a trace in the liver). The liver of one specimen of *Æolidia* contained 0·0008 per cent. while there was none in the livers of three others, all from Millport. *Pecten* showed traces in the livers of five individuals from Millport and of one from the Eddystone, while it was absent from the same organ of two from the Mewstone. *Anodonta* from Newdigate had traces in liver and kidney while specimens from Liverpool and Sheffield were devoid of nickel. It appears that in molluscs nickel is most often found in the liver, but that it may be either present or absent (in detectable quantities) in different members of one and the same species.

We have not found cobalt nearly so often as nickel. All our cases are in marine animals. Table IV lists all recorded cases of cobalt and all specimens containing over 0·002 per cent. of nickel.

Table IV.—Occurrence of Cobalt and of High Percentages of Nickel.

Specimen.	Animal.	Cobalt.	Nickel.
		per cent.	per cent.
10	<i>Myxicola</i>	0·002	about 0·01
76	"	0·002	0·008
139	"	0·002	0·008
406*	"	0·002	0·01
407	"	0·002	0·009
432	<i>Aphrodite</i> , body wall	0·0025	0·003
106	<i>Pinna</i> , kidney	0·006	0·005
116	<i>Aplysia</i> , liver	trace	0·004
130	<i>Haliotis</i> , epipodium	absent	0·004
376	<i>Archidoris</i> liver	0·0034	absent.
379	" "	0·0031	"

* Plate 8, No. 9.

The table shows that all high percentages of nickel were accompanied by cobalt, except in the case of *Haliotis*. *Archidoris*, on the other hand, has cobalt with no nickel. Here there is selective absorption of the former element. Neither nickel nor cobalt seem to have been recorded from sea-water.

With such an irregular incidence of nickel and cobalt, it is difficult to imagine that they have a function in the organism, but Bertrand and Nakamura (1927) raised 14 mice on a diet lacking nickel and cobalt, and 15 on a diet containing these elements, and found that the animals which had received the metals had an average length of life 17 per cent. greater than those without them. Again, Bertrand and Mâchebœuf (1926), finding that the pancreas of mammals contains both nickel and cobalt (Ni, 0·00000041 per cent., and Co, 0·00000069 per cent. dry weight), went on to show that nickel and cobalt added together to insulin prolong the destruction of sugar by this insulin in animals with diabetes. Cobalt (like zinc and mercury) in the right dose stimulates the growth of *Aspergillus* (Burkewitsch and Orlow, 1922).

6. Lead.

This is another element with an irregular and spasmodic distribution. It is well known that in cases of lead poisoning the element can be stored up in certain tissues. We have found lead in the three human subjects studied. The remarkable fact emerged that the lead was present in different organs in each man. One had lead in heart muscle, spleen, testis, and cartilage, but none in pancreas or bone. A second showed lead in pancreas and bone but none in the organs in which it was found in the first subject. A third had lead only in the liver and brain.

Earthworms and snails demonstrated that lead is present to varying extents in animals from different localities. Below is given the occurrence of lead in the posterior thirds of individuals of *Lumbricus* from various localities. The symbols have the following meanings: ++, lead abundant; +, present; tr., a trace; 0, absent.

Cambridge	0	0	0
Liverpool	0	tr	+
London	0	0	0
Marlborough	0	+	+
Sheffield	tr		

The distribution of lead in the livers of *Helix aspersa* was as follows (cf. Plate 8, Nos. 11, 12):—

Cambridge	+	+	+
Liverpool	0	tr	
London	tr	tr	
Marlborough	++	++	++
Plymouth	+	+	+

Lead was found in the kidney of *Pinna* and kidneys and livers of *Pecten*, but was absent from the remaining molluscs. It was present in *Aphrodite* (body wall) and *Spirographis*, but absent in the 17 other polychaetes. Plate 8, No. 5, shows lead in the medusa *Cosmetira*.

Apparently, then, this element is taken up when it is available and when tissues have an affinity for it. But it is puzzling why in different individual men different tissues should absorb the lead. One cannot suppose that with such an irregular distribution lead has a function in animals, but it may have effects as yet unknown.

Lead occurs in sea-water to the extent of 0.00004 per cent. and has previously been recorded in crustacea and molluscs (Chapman and Linden, 1926; no separate organs studied).

7. Silver.

Silver has previously been recorded from a coral (Forchhammer, 1865), from ascidians (Azéma and Pied, 1930) and from mushrooms (Ramage, 1930). We find that, like lead, silver has a wide, if spasmodic, distribution in animal tissues.

Of the three human subjects studied, one had silver in all organs and tissues, while this element was completely lacking in the other two.

Among the polychaetes silver was present in *Owenia*, *Melinna*, *Myxicola* (all five specimens, cf. Plate 8, No. 9), and *Sabellaria* (1 out of 8 specimens). It was not found in the remaining 15 species. There was no silver in any of the *Lumbricus*.

Silver was present in the blood of *Maia* and *Carcinus*.

In *Helix aspersa* silver was present in 11 out of the 13 livers (cf. Plate 8, No. 12). The greatest concentration was 0.001 per cent. Silver was in the foot of three out of 5 specimens of this species, and in 2 shells out of 10. The only silver found in *H. pomatia* was in one shell out of three. *Arion* contained none, nor did *Anodonta*.

Among the marine molluscs silver was absent only from *Patella* and *Æolidia*. *Haliotis* had silver in the liver, and *Archidoris* in the body wall of 1 out of 4 examples, and in the stomach of 1 out of 2. The secretion of *Aplysia* containing aplysiopurpurin had silver in it (Mn, Cu, Li and Sr were also present). The liver of *Ostrea* showed silver, and *Pecten* (Plate 8, No. 10) contained the element in 8 liver specimens (greatest concentration 0.005 per cent.), and in one kidney out of three. The strongest lines of silver were given by the liver and kidney of *Pinna*. Mantle, ovary and byssus of this animal also

contained silver. In *Sepia* it was present in liver, vas deferens and shell, and in *Loligo* in the liver.

Sea-water contains 0·00001 per cent. of silver (Liversidge, 1895).

8. Cadmium.

There seems to be no record of cadmium in any living organism, although zinc is known to be widely distributed. We have found cadmium in the livers of all specimens of *Pecten* (Plate 8, No. 10). The quantity varied from 0·05 to 0·20 per cent. Cadmium is not known in sea-water.

9. Lithium.

Lithium has been recorded from milk and human tissues (Kirchhoff and Bunsen, 1861; Hermann, 1905), from teeth and bones of the pig and man (Desgrez and Meunier, 1927), and from the hen's egg (Bishop, 1928). We have found that this element is very widely distributed in animal tissues (see Plate 8).

Lithium was present in all the marine animals examined; *Clathrina*, for example, contained 0·005 per cent. The highest concentration found in the polychætes was 0·0008 per cent. in *Melinna*, *Notomastus*, *Arenicola*, and the scales of *Aphrodite*. In *Pecten* 0·0002 per cent. was present in the mantle and gills and 0·00008 per cent. in liver. Lithium was found in 17 out of 19 plankton catches, usually in considerable concentration, which reached 0·002 per cent. in one specimen (153). The element has been detected in sea-water (Thorpe and Morton, 1871; Dieulaufait, 1879).

The fresh-water mussel *Anodonta* forms a striking contrast to the marine molluscs. Lithium was absent from all organs of the four individuals from Liverpool and Sheffield. It was present only in the mantle, gills and kidney of animals from Newdigate. Here presumably is a variation in lithium content with the supply.

Among the land molluscs, lithium was present in all the organs of *Arion*, and of *Helix aspersa*, and in all the organs of *H. pomatia* except the kidneys. There was lithium in all *Lumbricus* examined.

Lithium was found in skeletal muscle, heart muscle, bone and cartilage, but not in the other tissues, of one human subject, while it was present in the bone only of another. It was present in a human tooth.

Lithium, then, is a very widespread but, in the concentrations detectable by us, not a universal constituent of protoplasm. It varies in amount in a given tissue in different individuals.

10. *Rubidium*.

Rubidium was found to be present in all the organs of the three human subjects. The greatest concentration, 0.006 per cent., existed in the testis of one subject. This evidence, taken alone, suggests that rubidium may be a constant constituent of protoplasm, like iron and copper. Apparently, however, such is not the case, for in the invertebrates the lines of this element appear only in relatively few spectra.

Among the polychaete worms, only two species out of the total 19 were found to contain rubidium. These were *Notomastus* (0.0014 per cent., Plate 8, No. 7) and *Myxicola* (a trace in 1 out of 5 individuals).

Among the molluscs rubidium was present in the following instances: *Arion* (body wall, liver and gonad, each with 0.002 per cent.), *Archidoris* (female mucus gland, 0.002 per cent. in one individual, a trace in another, absent in two), *Eolidia* (mucus gland, 0.002 per cent.), *Sepia* and *Loligo* (a trace in the testis of each).

The quantity of rubidium in tissues can vary with locality. *Helix aspersa* demonstrates this as follows:—

Cambridge and London		None in any organs.
Liverpool		Two specimens with 0.0008 per cent. in the kidneys.
Marlborough		Traces in foot, kidney and dart sac.
Plymouth		0.002 per cent. in the liver of one (Plate 8, No. 12), traces in the livers of two, and traces in foot, kidneys, com. gen. duct, albumen gland, and dart sac.

A similar variability is shown by *Lumbricus*. In specimens from Liverpool and Marlborough, rubidium is present, while it is absent in those from Cambridge, London and Sheffield.

It is noticeable that rubidium is absent in both *H. pomatia* and *Lumbricus* from Cambridge and London and present in both of these animals from Liverpool and Marlborough. This looks as if the element were absorbed by protoplasm of quite different animals in proportion to its availability.

Rubidium has previously been recorded from plant ash (Grandeau, 1863; Robinson, 1918; Ramage, 1930) and from human organs, human milk and cow's milk (Ramage, 1929). It is present in sea-water to the extent of 0.04 per cent. of dry salts (Clarke, 1924).

Our method allows of the detection of caesium, the sensitivity of the test being equal to that for rubidium. Yet caesium was not found in any of our specimens. It is known that caesium is present in sea-water and in oyster shells (Sonstadt, 1870) and it has been detected in plant ash (Robinson, 1918). Frog muscle absorbs both rubidium and caesium from perfusion fluid (Mitchell, Wilson and Stanton, 1921).

11. Strontium.

Strontium has up to now been recorded only from a radiolarian, *Podocaneilus* (Bütschli, 1908), from "the calcareous parts of marine organisms" (Dieulauf, 1877), and from mammalian blood, teeth and bones (Desgrez and Meunier, 1927). We find that this element is very common in animal tissues (see Plate 8).

The lines of strontium appeared in 17 out of the 19 species of polychaete worms. Those showing no strontium were *Nephtys* and *Notomastus*. The highest concentration, 0.002 per cent., was in *Protula*.

Ten species of marine molluscs contained strontium. It was present in 51 out of the 67 different organs and tissues examined from these animals. The highest concentrations were as follows: more than 0.008 per cent. in *Limacina*, the foot of *Aplysia*, the body wall of *Archidoris* (4 examples), and the ink sac and shell of *Sepia*; 0.008 per cent. in the egg-shell of *Sepia*; 0.006 per cent. in the foot of *Halotis*; 0.004 per cent. in the blood of *Aplysia*, and in the secretion containing aplysiopurpurin; 0.002 per cent. in the eggs of *Aplysia*.

In the fresh-water mussel *Anodonta* from Newdigate strontium was present in all organs except the foot, but in examples from Liverpool it was found in the shell only. There is thus a variation in strontium content in different localities.

Strontium is very widespread in snails. It was present in all organs of *Helix pomatia*. The same was the case of *H. aspersa* from Marlborough. The element was absent only from one or two organs of members of this species from the other localities. The amount of strontium in the common genital ducts of *Helix* was curious. In *H. pomatia* the concentration here was very high, being over 0.008 per cent., while in *H. aspersa* there was less than 0.0008 per cent. (cf. Plate 8, Nos. 13, 14).

Strontium was present in high concentration in *Clathrina*, *Beroë* and *Sagitta* and was found in gonads, gut, and body fluid of the echinoids, and in the blood of *Maia* and *Carcinus*. It was absent from insects, spiders and the centipede. The only strontium found in man was a trace in a tooth.

Both strontium (Dieulafait, 1877) and barium (Harvey, 1928) occur in sea-water. No barium was found in any of our specimens, but our method of analysis is less sensitive for barium than for strontium in the ratio of 1/25 for the green line, which is usually masked by calcium, and not less than 1/125 for other barium lines as compared with the blue strontium line. Further, as far as concerns the marine animals, the quantity of barium in sea-water is probably much less than the quantity of strontium.

12. Fluorine.

Fluorine was recorded by Nicklès (1856) in the bones and blood of man, in the blood of mammals and birds, and also in bile, saliva, urine, hair, and egg-albumen. Carles (1907) found fluorides to the extent of 0.00002 per cent. in the shells of *Helix*, *Limnæa* and *Planorbis*, 0.00003 per cent. in those of fresh-water mussels, 0.012 per cent. in those of oysters and marine mussels, and 0.0012 per cent. in sea-water. Gautier and Clausmann (1914) give 0.00003 as the percentage of fluorine in sea-water.

The only animal in which we detected fluorine was *Archidoris*. The band of calcium fluoride was strong in the body wall of all four examples examined (Plate 8, No. 8).

13. Summary.

(1) The present paper is the first report on a quantitative spectrographic analysis of animal tissues. It deals largely, but not exclusively, with annelids and molluscs.

(2) Iron and copper were present in all tissues analysed.

(3) Manganese was widely distributed. The manganese content of tissues varies with locality. Its function is discussed.

(4) Nickel and cobalt occurred spasmodically, the former being more frequent. Except in one case, all high concentrations of nickel were accompanied by cobalt. In one tissue only did cobalt occur without detectable nickel.

(5) Lead and silver both exhibit an irregular distribution. Certain tissues have a disposition to accumulate one or other of these elements. One man had silver in all organs, two others had none. Lead accumulates in different tissues of different individual men.

(6) Cadmium occurred in the livers of *Pecten maximus* from several different situations.

(7) Lithium is very widespread in animal tissues, rubidium is much less so, while caesium was not found.

(8) Strontium was found in numerous cases. Barium was not detected.

(9) Calcium fluoride was found in one case only, namely, the body wall of *Archidoris tuberculata*.

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The Adsorption of Substances by Fuller's Earth.

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(Abstract.)

Propionic and hexoic acids are not adsorbed by fuller's earth to any extent at any p_H . Oxalic acid is adsorbed in solutions more alkaline than p_H 1.5. The adsorption rises to a maximum at about p_H 4 and then falls off very gradually with increasing alkalinity. At p_H 12 the adsorption is about three-quarters of its maximum value.

The adsorption of *n*-propylamine and *n*-butylamine is influenced by acidity in almost exactly the same way. In both cases the adsorption rises from a vanishingly small value in very acid solution to about p_H 4.5. From p_H 4.5 to about p_H 8 the adsorption is almost independent of the hydrogen-ion concentration. In solutions more alkaline than p_H 9.5 there is a very rapid rise in the adsorption to a maximum at p_H 11, which is immediately followed by a rapid fall to a vanishingly small value at p_H 13.

It has been found that between p_H 4.5 and p_H 8, over which range the adsorption remains almost constant, an amount of calcium is displaced which is almost exactly chemically equivalent to the base adsorbed. In more alkaline solutions, the calcium displaced falls to zero. It is concluded, therefore, that the adsorption of these bases by fuller's earth is a true apolar process in very alkaline solutions, but that in solutions more acid than p_H 8 adsorption proceeds almost entirely by the displacement of calcium ions from the fuller's earth.

Further evidence in support of this view has been obtained by studying the adsorption of *n*-butylamine by aluminium silicate gel. In this case there is no calcium to be displaced. The adsorption of the base rises from nil at p_H 7 to a sharp maximum at p_H 11, followed by a rapid fall in more alkaline solution.

The fact that aluminium silicate gel does not adsorb oxalic acid at all at any hydrogen-ion concentration suggests that the adsorption of this acid by fuller's earth is due to the formation of insoluble calcium oxalate at the surface.

In conclusion I would like to express my sincere thanks to Professor R. A. Peters for his kind interest and helpful advice throughout this work, and to express my indebtedness to the Ramsay Memorial Trustees for a Fellowship which placed me in a position to carry out these researches.

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The Effect of Light on the Oxidation of Fats.

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It has long been known that exposure to light exerts a powerful influence upon the deterioration of fats, and it has even been claimed that light alone, in the absence of air, is capable of producing rancidity (Wagner, Walker and Oestermann, 1913). It is now, however, generally admitted that the action of light consists essentially in accelerating oxidation of the fat. To follow the changes induced in fats by exposure to air and light it is necessary (1) to have a quantitative knowledge of the state of oxidation in any specimens of fat; (2) to be able to detect and follow the very earliest stages in the process of oxidation; and (3) to possess the means of comparing the potential keeping qualities of any two or more fats. Since no existing methods satisfy these requirements a new test for rancidity has been devised. (Lea, 1929).

A New Test for Rancidity.

Briefly, the process consists in heating the oil or fat with a solvent mixture of glacial acetic acid and chloroform in the presence of solid potassium iodide. Oxidation of the reagent or fat during the process is prevented by previously filling the tube with nitrogen, while the steady evolution of chloroform vapour effectively prevents diffusion of oxygen back into the tube. The reaction mixture is cooled, poured into 5 per cent. potassium iodide, and titrated with N/500 sodium thiosulphate solution.

The sensitivity of the method is such that using 1 g. of fat a content of active oxygen equivalent to 0.1 c.c. of N/500 thiosulphate solution, i.e., 1.6×10^{-6} g. of oxygen, can be detected. Owing to loss of iodine by combination with the fat the amount of active oxygen measured is always slightly less than the amount actually present. This point together with experimental details of the method is discussed in full in Appendix I.

In addition to the test for active oxygen outlined above, a modification of the Kreis test has also been used. A determination is carried out as follows.

The fat (1 g.) is dissolved in benzene (2 c.c.), concentrated hydrochloric acid (A.R., 1 c.c.) added and the mixture shaken for one minute. A 0.1 per cent. solution of phloroglucinol in ether (1 c.c.) is then added, shaking continued for a further minute, and after separation by means of the centrifuge the pink aqueous layer is compared with a set of permanganate standards. These are prepared in the following manner. Potassium permanganate (0.2 g.) is dissolved in distilled water, acidified with dilute sulphuric acid and made up to a litre (solution No. 5). Five successive dilutions (Nos. 4, 3, 2, 1 and 0) are prepared from this solution diluting 2.5 times at each stage. The standards are renewed as soon as manganese dioxide begins to be deposited. To determine the intensity of the Kreis test, "n" is first estimated by direct comparison with the standards. The intensity is then taken to be $(2.5)^n$. It is probable that this method interprets the eye's estimate of the intensity rather better than does the use of simple arithmetical proportion; the difference between the two sets of figures, however, is usually not great. With pure fats consistent results can be obtained with fair accuracy, but in the presence of appreciable quantities of non-fatty material the test becomes much less satisfactory. It is essential that the ether used in making up the phloroglucinol reagent be free from peroxide, otherwise extremely erratic results may be obtained.

Finally the Schiff reaction has been used but in a form somewhat modified in order to make it easier of application to solid fats (see Appendix 2).

Determination of the Relative Susceptibilities of Fats to Oxidation.

The samples of fat are illuminated under similar conditions and the active oxygen content determined at intervals. In the present work use has been made of a constant temperature room at 25° C., the fat being placed at a distance of 3 feet below the filament of a 100-watt gas-filled lamp (with shade). Small flat-bottomed, crystallising dishes 5 cm. in diameter, each containing 3.5 or 5 g. of fat, are used. Sixteen dishes may be irradiated by one lamp, e.g., four dishes of each of four samples of fat. A dish is removed at intervals for analysis.* By screening the lamps and by the use of partitions several experiments may be carried out simultaneously in the same room. New lamps

* The maximum difference in intensity of the light received by any two dishes is less than 2 per cent., the intensity of illumination being taken as proportional to $1/D^2 \cos \theta$, where D is the distance between the lamp filament and the centre of a dish, and θ the angle of incidence. This error may be minimised by using the dishes furthest from the centre first, while the titration is still low.

of the same batch can usually be relied upon to give fairly uniform results. It is unsatisfactory to replace the lamps by diffused daylight or sunlight, since the stronger illumination, by greatly reducing the induction period, masks the differences in susceptibility between the various specimens.

The Effect of Various Intensities of Illumination.

The order of magnitude of the influence of light from different sources may be gathered from fig. 1 and Table I.

In compiling the table use has been made of the approximate temperature coefficient of 1.83 obtained from experiments on cod-liver oil (*cf.* p. 185).

Table I.

	Darkness.	3 feet from 100-watt lamp.	Winter.		Summer.	
			Sky light through glass.	Sunlight.	Sky light through glass.	Sunlight.
Temperature	° C. 15	° C. 15	° C. 17	° C. 23	° C. 25	° C. 35
Relative effect	0.02-0.05	1	28	162	64	470
Reduced to same temperature	0.02-0.05	1	25	100	35	140

Under the influence of strong light it was found that the pronounced induction period observed in the dark and under relatively weak illumination became reduced almost to vanishing point. This was demonstrated as follows. Beef kidney fat* was extracted from the tissue with petroleum ether, powdered (*cf.* p. 183), and exposed on glass plates in a room with sky lighting (not direct sunlight) during dull weather in early February. The course of the resulting oxidation is shown in fig. 5 (iii). Fig. 4 (iii) shows the curves obtained from similarly extracted beef external fat exposed in small flat-bottomed dishes (5 cm. diameter, 5 g. per dish) to a July sky in the same room. The effect of direct summer sunlight was still greater. Specimens of minced beef kidney fats (A and B) which were exposed to direct sunlight on hot July days—mean temperatures 34° C. and 37° C. respectively—in 6 hours had reached a content of active oxygen corresponding to 50 c.c. of N/500 thiosulphate solution per gram.

* The expression "beef kidney fat" is used in this paper to denote the perirenal fat, *i.e.*, the mass of adipose tissue surrounding the kidneys.

When the minced or powdered fat was spread on glass plates the temperatures given are those registered by a thermometer with bulb lying in the fat. When the fat was contained in small dishes the thermometer lay on the table beside them. It follows that the temperature recorded for artificial and diffused daylight will be fairly accurate, but for exposure to sunlight they may be inaccurate owing to local heating of the fat.

To obtain a quantitative measure of the relation between rate of oxidation and intensity of the light, a sample of fresh beef kidney fat was minced finely at 0° C. and spread in a thin even layer on flat glass plates which were placed at various distances below a 100-watt gas-filled lamp suspended near the ceiling of a dark room. As a control experiment two similar plates were kept in the same room but completely screened from light. Since the fat consisted of small cylinders arranged at random but with the long axes mainly horizontal, the intensity of illumination was taken as approximately proportional to $(1 + \cos \theta)/2D^2$, where D is the direct distance from the lamp filament, and θ the angle of incidence. Table II gives the active oxygen content (expressed as cubic centimetres N/500 thiosulphate solution per gram of fat) for different light intensities at the end of 260 and 400 hours.

Table II.—The Influence of Light Intensity on the Velocity of Oxidation.

Experiment number.	Mean temperature.	Light intensity.	Active oxygen content.	
			260 hours.	400 hours.
	° C.			
1	20.5	100	5.10	22.68
2	20	53.7	3.30	12.99
3	20	27.6	2.12	6.80
4	20.5	15.2	2.00	7.30
5	19.5	11.3	1.62	5.75
6	19.5	10.5	1.92	6.50
7	18.75	3.4	1.70	5.55
8	18.25	2.0	1.55	4.60
Control	19.5	0	0.17	0.33

Comparing experiments No. 1 and No. 8 it is apparent that reduction of the light intensity 50 times has only reduced the resultant oxidation by about five times. It follows that even very weak light may exert an appreciable influence on the rate of oxidation of fats.

The Autocatalytic Nature of the Oxidation.

The form of the curves in fig. 1 shows that the process is autocatalytic. It therefore seemed of interest to discover what would be the course of the oxidation after removal of the exciting light, when presumably a certain amount of

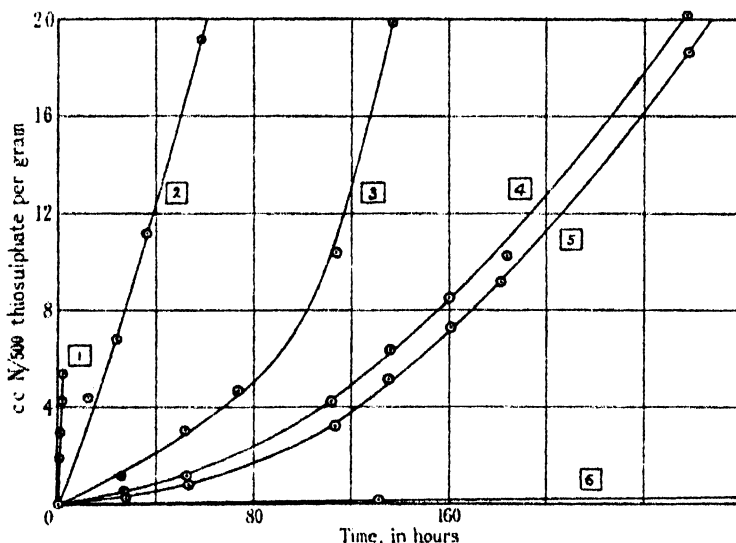


FIG. 1.—The effect of various intensities of illumination on the oxidation of minced beef kidney fat. (i) Direct sunlight, early March. Temperature 23° C., falling to 20° C. (ii) Diffused daylight, north window, early March. Mean temperature 15° C. (iii) Dark room, 1 foot 11 inches from 100-watt gas-filled lamp. Temperature 22° to 25° C. (iv) Dark room 4 feet from 100-watt gas-filled lamp. Temperature 20° to 23° C. (v) Dark room, 6 feet from 100-watt gas-filled lamp. Temperature 20° to 23° C. (vi) Unilluminated fat.

the autocatalyst generated by the action of the light would be present. For this purpose a specimen of minced beef kidney fat was spread on flat glass plates (4 inches square, 12 g. per plate), and exposed in a constant temperature room (25° C.) to the light from a 100-watt gas-filled lamp suspended 3 feet above it. Two lamps were used, each illuminating four plates. At intervals a plate was removed, sampled for analysis, and the residual fat placed in a large light-proof chest in the same room. The results are plotted in fig. 2, curves 1 to 9 representing the course of the oxidation taking place in the absence of light.

It will be seen that once oxidation has set in, removal of the source of light does not reduce the rate to that of the unexposed fat, but that the reaction proceeds in the absence of light at a rate depending upon the amount of active

oxygen already present. By plotting the gradients of curves 1 to 9 against the initial contents of active oxygen it is apparent that the catalytic effect produced

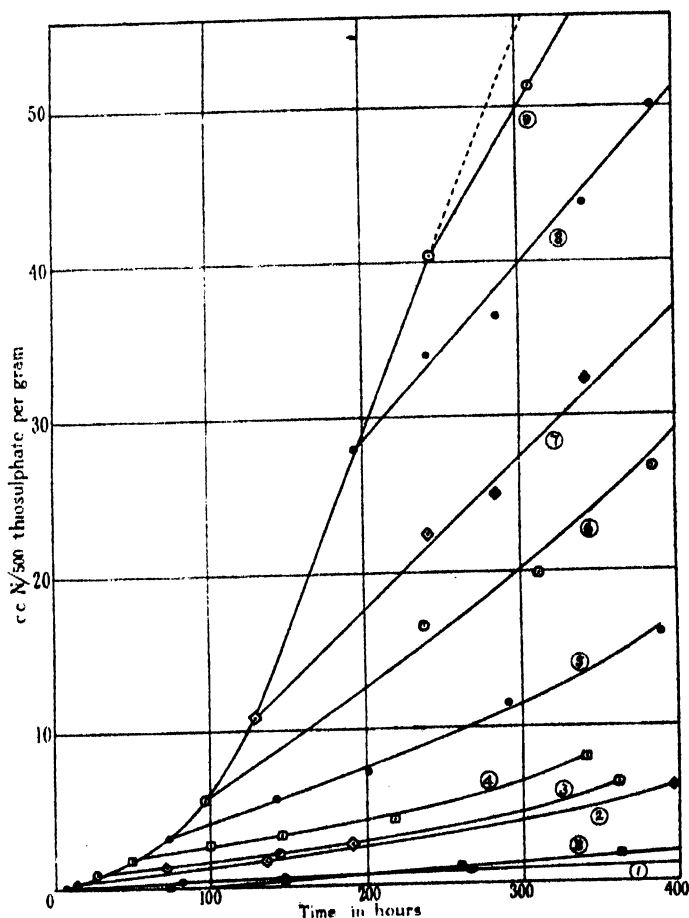


FIG. 2.—The effect of various periods of illumination in promoting the subsequent oxidation of beef kidney fat.

by quantities of oxygen up to about 6 c.c. N/500 thiosulphate per gram is directly proportional to the amount present. Between 6 c.c. and 10 c.c. the increase in catalytic effect for each cubic centimetre of oxygen added rapidly falls, becoming more or less constant above 10 c.c. at a fraction of its former value. The beginning of the approximately linear part of the oxidation curve depends to some extent upon the physical state of the fat. Values of roughly 8 c.c. and 15 c.c. were obtained respectively for powdered fat (1 g. per 14 sq. cm.) and a flat surface of solid fat 2.25 mm. thick (1 g. per 4 sq. cm.).

It follows from the autocatalytic character of the change that the presence of

quantities of active oxygen too small to exert any influence on odour and flavour may yet be very harmful from the point of view of keeping quality. It is therefore probable that the potential storage life of many fatty products is seriously impaired by the methods employed in their manufacture. To quote an instance. A sample of absolutely fresh rendered lard, obtained from a local factory, though sweet to the taste, already gave oxygen and Kreis values of 3.5 c.c. and 3.2 units respectively. The induction period of this fat exposed in flat-bottomed dishes (5 cm. diameter, 5 g. per dish) in a room at 25° C. to the light from a 100-watt lamp at 3 feet distance was extremely short (fig. 3 (i)). For purpose of comparison the oxidation curves obtained under similar conditions from a more resistant fat (fresh mutton kidney fat) are also shown.

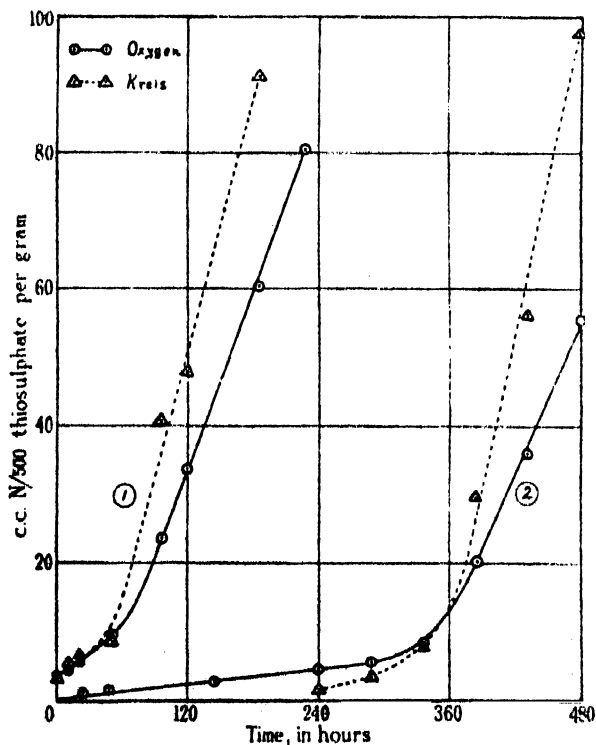


FIG. 3.—(i) Lard. Temperature 27° C. (ii) Mutton kidney fat (extracted). Temperature 27.2° C.

The very short induction period possessed by a partly-oxidised fat is again seen in fig. 6 in the case of cod-liver oil.

Differences in Susceptibility to Oxidation.

As might perhaps be expected, the readiness with which the fat undergoes oxidation may vary considerably in different animals. This is clearly shown in fig. 4. Two samples of fresh beef kidney fat from different animals were chopped finely on a glass plate at -5°C ., sieved, and the powdered fat spread in a thin uniform layer on glass plates (10 inches by 5 inches, 20 g. per plate). The two plates were placed in a constant temperature room at 25°C . and illuminated simultaneously by the same lamp suspended 3 feet above them. The respective rates of oxidation are plotted in curves (i) and (ii).

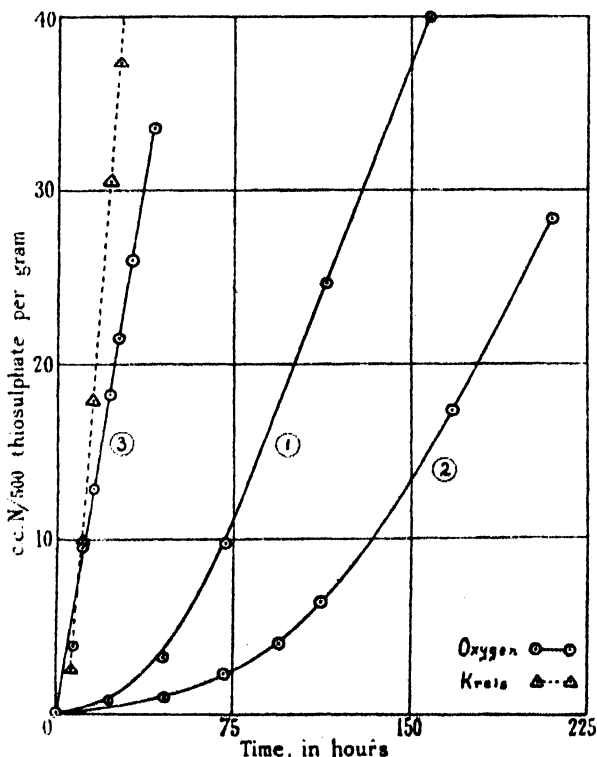


FIG. 4.—(i) Beef kidney fat A. Temperature 27.1°C . (ii) Beef kidney fat B. Temperature 27.1°C . (iii) Beef external fat (extracted). Summer sky light. Mean temperature 25°C .

Beef kidney fat is a complex containing in addition to the fat proper (*circa*. 96 per cent.) smaller quantities of water (*circa*. 3 per cent.), connective tissue (dry weight < 1 per cent.), blood, pigment, sterols, etc. Robinson (1924) has shown that hæmoglobin accelerates the oxidation of drying oils. Moisture is usually said to favour the oxidation, but according to Holm and Greenbank

(1924) retards it. It was therefore quite possible that the observed differences in susceptibility of these two fats might be due to variation in composition or amount of the non-fatty constituents, rather than to actual chemical diversity in the fats themselves. The two samples were therefore ground up with anhydrous sodium sulphate and silver sand, and extracted with peroxide-free ether using a hot extraction apparatus in a darkened room. After removal of the solvent under vacuum the fats gave the following analyses :—

Fat.	Iodine number.	Sap. equivalent.	Free acidity (as percentage oleic).	Colour of the melted fat (as percentage N/100 $K_2Cr_2O_7$).
A	42.51	285.2	0.24	30
B	40.25	284.0	0.18	11

The extracted fats were now chopped, spread on glass plates, and irradiated under conditions similar to those employed in the previous experiment. The

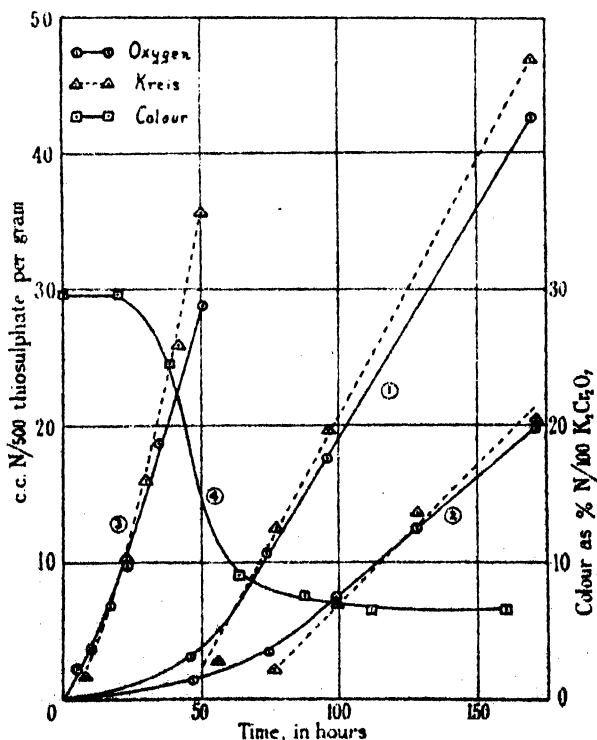


FIG. 5.—(i) Beef kidney fat A (extracted). Lamp. Temperature 27.3°C . (ii) Beef kidney fat B (extracted). Lamp. Temperature 27.3°C . (iv) Beef kidney fat A (extracted). Lamp. Temperature 27.3°C . (iii) Beef kidney fat B (extracted). Winter sky light. Mean temperature 17.2°C .

result expressed graphically in fig. 5 (i) and (ii) shows that the general difference between the two fats still held.

The difference in susceptibility of these two fats cannot be attributed to moisture, connective tissue, enzyme or blood content, but must, in the main, be due to their chemical nature. The possibility that the fat pigment may play some part in the oxidation must not, however, be forgotten. No attempt was made to correlate susceptibility with age, food, breed, etc., of the animal.

The Bleaching Action of Light.

It is well known that the pigments present in natural fats often may be destroyed by exposure to light, in fact this principle has been applied technically in the refining of oils. In order to investigate the relationship between colour change and the general oxidation process a rather deeply-coloured specimen of beef kidney fat (A) was used. The extracted fat was powdered, spread on a glass plate, and exposed as usual in a constant temperature room at 25° C. to the light from a 100-watt lamp. The active oxygen content was determined at intervals, and in addition samples each of about 3 g. were removed, rapidly melted, and the colour compared with a series of tubes containing known dilutions of N/100 potassium bichromate solution. The results are plotted in fig. 5 (iv). It is evident that by the time the linear part of the oxidation curve is reached bleaching is already complete. The solid finally obtained was pure white and lard-like in appearance, quite different from the original yellow fat.

Intensity of the Kreis Test in Relation to Active Oxygen Content.

As already mentioned, in addition to the test for active oxygen, use was made of the Kreis reaction to follow the oxidation of fats under the influence of light. It has been stated by Holm and Greenbank (1923) that the intensity of the Kreis test is directly proportional to the amount of oxygen which has been absorbed by the fat. From the preceding diagrams (figs. 3, 4 and 5) it will be seen that in the present experiments a distinct parallelism has been found between the "active" oxygen content of a fat and the intensity of the Kreis reaction.* This relationship, however, is not constant, but may vary greatly under different conditions. At low temperatures the rate of increase of the Kreis reaction is much smaller than that of the content of active oxygen, but in bright sunlight the Kreis reaction increases much the more rapidly.

* It should be noted, however, that the units in which values of the Kreis intensity are plotted are obtained from an arbitrarily chosen set of permanganate standards (cf. p. 181).

Fig. 6 represents an experiment carried out with crude cod-liver oil illuminated at three different temperatures by a 100-watt lamp at 3 feet distance.

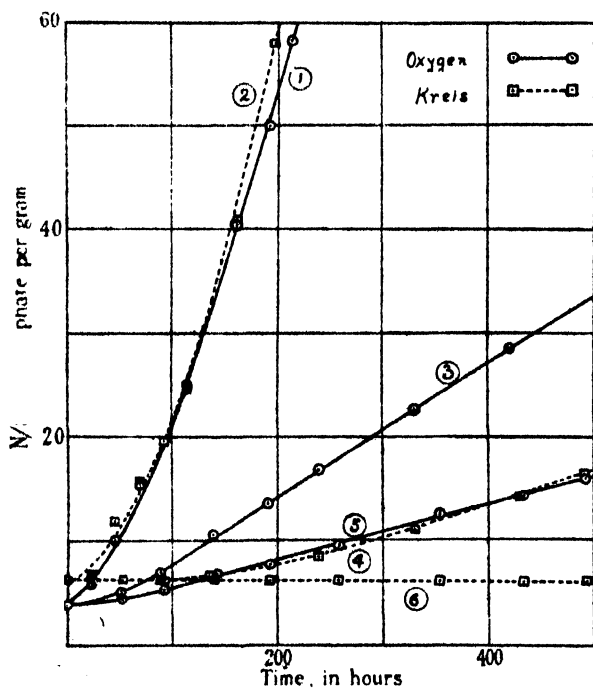


FIG. 6.—The effect of temperature on the Kreis-active oxygen relationship. (i) Active oxygen 27.5° C. (ii) Kreis reaction 27.5° C. (iii) Active oxygen 7.4° C. (iv) Kreis reaction 7.4° C. (v) Active oxygen -9.4° C. (vi) Kreis reaction -9.4° C.

The temperature coefficient for the accumulation of active oxygen between 7.4° C. and 27.5° C. is approximately 1.83, that for the Kreis reaction considerably higher.

The Influence of Oxidation on Free Acidity.

Oxidation in the presence of light appears to have little effect, at least in the earlier stages, upon the free acidity of the fat. No detectable increase could be observed for low contents of active oxygen, while titrations up to 60 c.o. of N/500 thiosulphate solution per gram resulted only in increases of the order of 0.2 to 0.3 per cent. (calculated as oleic acid).

Summary.

A quantitative test for the detection of oxidation in fats has been described, together with a technique for quantitative application of the Kreis reaction.

A new method for determination of the relative susceptibilities of fats to oxidation has also been outlined.

Oxidation is accelerated sensibly by weak artificial light, while exposure for a few minutes to direct sunlight is sufficient to produce rancidity.

The reaction is autocatalytic and even brief exposure to light accelerates the subsequent oxidation.

The existence of differences in susceptibility between various specimens of beef fat has been pointed out, and the effect shown to be due to the chemical nature of the fat rather than to the non-fatty material present.

Bleaching of the yellow pigment of beef fat has been shown to occur at a comparatively early stage in the oxidation process.

The relationship between intensity of the Kreis test and active oxygen content of the fat has been found to vary with the temperature.

APPENDIX 1.

The Active Oxygen Test.

Results obtained in this test depend upon the proportions of fat and solvent employed. When too high a concentration of fat is used results are low owing

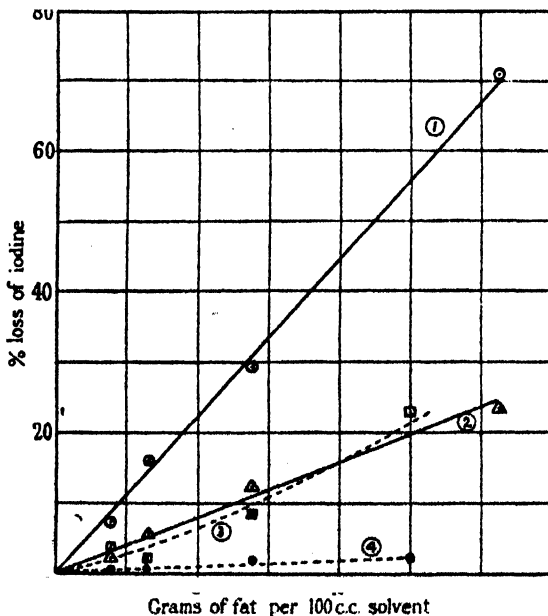


FIG. 7.—(i) Cod-liver oil and (ii) beef kidney fat. Each containing active oxygen equivalent to 40 c.o. N/500 thiosulphate per gram. (iii) Cod-liver oil and (iv) beef kidney fat. Unoxidised; in the presence of benzoyl peroxide equivalent to 40 c.o. thiosulphate per gram of fat.

to absorption of iodine, particularly in the case of a highly unsaturated fat. The magnitude of this effect has been investigated in the case of beef kidney fat (iodine number 42·5) and cod-liver oil (iodine number 177·6). A specimen of each fat was oxidised in a constant temperature room under the influence of light from a 100-watt lamp. At intervals a sample was removed and the apparent active oxygen value determined using 1 g. of fat in 4, 9, 19 and 32·3 c.c. of solvent. Fig. 7 (i) and (ii) shows the effect of concentration on the absorption of iodine, when the true active oxygen content of the fat (obtained by extrapolation to zero concentration) was in each case 40 c.c. N/500 thiosulphate solution per gram.

Using 1 g. of fat in 19 c.c. of solvent it is evident that the fraction of the total peroxide measured is about 95 per cent. in case of beef kidney fat and 85 per cent. in that of cod-liver oil. For other contents of active oxygen the figures obtained were as follows :—

True active oxygen content (c.c. N/500 thiosulphate per gram).	Percentage measured (1 g. fat in 19 c.c. solvent.)	
	Cod-liver oil.	Beef kidney f
40	85	95
20	86	95
10	89	96
5	92	98

Results obtained with any particular concentration of fat in solvent are quite definite and reproducible. No correction for absorption of iodine has been applied to any of the experimental results given in this paper.

In a further experiment, fig. 7 (iii) and (iv), 1 g. of fresh unoxidised fat was used in conjunction with different volumes of solvent, the iodine (equivalent to 40 c.c. N/500 thiosulphate solution) being provided in each case by the requisite quantity of benzoyl peroxide. It was possible by this means to determine directly the loss of iodine at each dilution. The absorption was now found to be much smaller than when the iodine was liberated by the fat peroxide. It is possible that addition of iodine to the double bonds of the fat occurs most readily at the instant of removal of the oxygen.

The presence of protein in not too large quantities, *e.g.*, animal fats in the tissue, etc., does not interfere with the determination except in that adsorption of iodine by the non-fatty material renders the end point in the titration somewhat slower and less definite.

The following materials are required for a determination, (i) a solvent mixture consisting of glacial acetic acid (A.R.) and chloroform (A.R.) in the proportion of 2:1 by volume; (ii) potassium iodide as solid (powdered A.R.) and as a roughly 5 per cent. solution prepared by dissolving the crystals in freshly boiled and cooled distilled water. The solution should be made up only as required; (iii) sodium thiosulphate solution (N/500) prepared by dissolving sodium thiosulphate crystals (A.R. 2.5 g.) and sodium carbonate (anhydrous 0.1 g.) in carbon dioxide free water (5 litres). The solution is kept in a syphon bottle fitted with a soda-lime guard tube and protected from light.

Standardisation is carried out as follows. To 10 c.c. of standard N/100 potassium bichromate solution, 5 per cent. potassium iodide (20 c.c.) and concentrated hydrochloric acid (A.R. 10 c.c.) are added, and the liberated iodine titrated at once. A blank is carried out using 10 c.c. of distilled water in place of the bichromate, and immediately after addition of the acid running in distilled water (boiled and cooled, 50 c.c.) from a burette. The standardisation should be carried out in artificial light. Under these conditions the blank does not usually exceed 0.3 to 0.5 c.c.

The apparatus used is simple. Strong thick-walled test tubes 17 mm. in diameter are required. The tubes are fitted with rubber stoppers, machine bored with one hole (*circa.* 4 mm. in diameter). A glass plug 8 mm. in diameter, 3 cm. long, rounded at one end and flattened at the other, and a nitrogen cylinder fitted with fine adjustment valve are also necessary.

A determination is carried out as follows. The fat (1 g.) is weighed into the tube, solid powdered potassium iodide (1 to 2 g.) added, and the solvent mixture (19 c.c.) run in from a graduated pipette, washing down any potassium iodide adhering to the walls of the tube. A rubber stopper is fitted, the tube supported in some type of stand in which it is protected from light, and a steady stream of nitrogen passed for two minutes into the air space above the liquid by means of a narrow glass jet which passes loosely through the hole in the stopper. The jet is then removed, the finger placed lightly over the hole, and the tube heated in an inclined position (rotating to prevent cracking) over a very small flame applied to the bottom of the tube. When the liquid is bubbling fairly freely the tube is plunged into a large boiling-water bath. The liquid rapidly boils, the chloroform vapour expelling the nitrogen and any remaining air. As soon as vapour is issuing from the hole, as shown by condensation on the edges, the finger is removed and a glass plug forced in. This operation should be carried out when the frothing liquid is nearly at the top of the tube. The closed tube is shaken vigorously for a few seconds and cooled

under the tap. The stopper is then removed, the liquid instantly poured into a conical flask (150 c.c.) containing 5 per cent. potassium iodide solution, the tube rinsed out twice with potassium iodide and the free iodine titrated. It is advisable to carry out the determination by artificial light. The author's room at the Low Temperature Research Station is fitted with dark blinds. Illumination is obtained from a single 60-watt lamp, and the boiling, cooling and pouring-out processes are carried out screened from direct light (the operator's own shadow will suffice). Titration is carried out with the aid of a daylight lamp, the end point being perfectly sharp and (in artificial light) displaying no tendency to "go back." A routine determination occupies roughly half an hour.

APPENDIX 2.

The Schiff Reaction.

The fat (1 g.) is weighed into a small tube (3 inches by $\frac{1}{2}$ inch, as used for the Kreis test), chloroform (1 c.c.) added and the tube warmed with shaking until solution is effected. The tube is then cooled and 0.05 per cent. fuchsin-sulphurous acid reagent (1 c.c.) added. The mixture is shaken for one minute and allowed to stand in a dark cupboard for 10 minutes. A milk-white emulsion indicates a negative reaction, and a violet colour a positive one. The test is of the same order of sensitivity as the Kreis reaction, but does not lend itself so readily to quantitative use.

The author wishes to express his thanks to Mr. C. R. Barnicoat, M.Sc., for the use of results in fig. 3, and to the Salters' Institute of Industrial Chemistry for a Fellowship during tenure of which this research has been carried out.

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*The Delayed Lethal Effect of Radium on Tissue Cultures in vitro—
Comparison of Continuous and Spaced Radiation.*

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Introduction.

In a previous paper (Spear, 1930) I have described the delayed lethal effects of radium on tissue cultures *in vitro*. Two quantities of radium were used in the experiments—100 mg. and 300 mg.—and exposures varied from 3 hours up to 24 hours. It was found that when a delayed lethal effect followed exposure to radium, it occurred sooner in those cultures which had been exposed to the greater intensity of radiation for a shorter time than in those subjected to the lesser intensity for a longer time, the product of milligrams of radium and hours of exposure being constant in a given comparison.

The following experiments were made to compare the length of the survival period of cultures exposed to a continuous irradiation known to be lethal, with the survival period following exposure to a spaced radiation of the same intensity.

Previous work showed that exposure of tissue cultures to 300 mg. radium at 0.5 cm. distance for 6 hours would produce a lethal effect within 16 days. It was decided to confirm this on a larger number of cultures than used hitherto, and then to compare the result with that following an exposure to the same radium for 1 hour daily on six successive days.

The expenses in connection with this study were met by a grant from the Medical Research Council. The radium used was lent, at the request of the British Empire Cancer Campaign, by Messrs. Watson & Sons and the Radium Belge.

Material and Methods.

Material.—The tissue was obtained from the choroid and sclerotic of fowl embryos of 8 to 9 days' incubation. Each culture was grown in fowl plasma and fowl embryo extract on a No. 1 coverslip inverted over a hollow ground slide, sealed with paraffin wax and incubated at 37° C.

Growths of the second subculture were used.

Radiological Technique.—Exposures to irradiation were made in a special

apparatus called a radium lantern, described in a previous paper (Canti and Spear, 1927). It consists essentially of two discs of commercial lead, each capable of carrying one hollow ground slide. The radium is arranged half-way between the lead discs so that two cultures can be exposed at one time.

The source of irradiation was 300 mg. radium element in the form of sulphate in a circular flattened platinum container with walls 0.5 mm. thick. The internal dimensions of the container were 13 mm. by 1 mm.

Specimens were brought from the culturing laboratory to a special room where the radium and lantern were kept at 37° C. After exposure the cultures were returned to the culturing laboratory, care being taken to prevent chilling during the transferences. Not more than six cultures were irradiated on any one day.

Cultural Technique.—(a) *Experiments with Continuous Irradiation.*—Immediately after irradiation specimens were subcultivated and returned to the incubator. Subcultivation was made every 48 hours so long as the specimen survived (see Chart 1). A culture was considered "dead" when, after 48 hours' incubation following the last subculture, it showed either no migration of cells or not more than three cells projecting from the body of the explant.

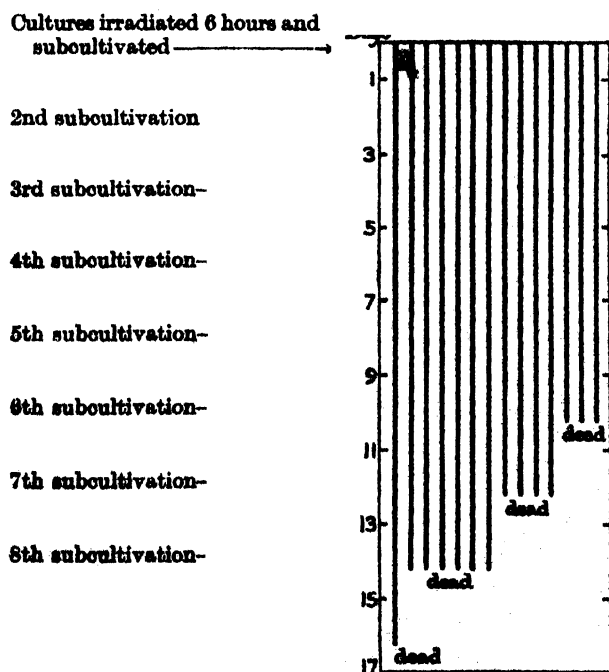


CHART 1.—Showing Survival Periods after Continuous Irradiations of 6 hours.
Each vertical line represents life history of one culture.

At subcultivation the explant was not divided but only "trimmed" and transplanted to fresh medium.

Control cultures were used to check the cultural conditions of the experiment, and these were not maintained after the death of the corresponding irradiated specimen.

(b) *Experiments with Spaced Radiation.*—Group I.—Cultures were irradiated for 1 hour daily on six successive days. They were subcultivated immediately after the first, third and fifth irradiation and every 48 hours thereafter so long as they survived (see Chart 2).

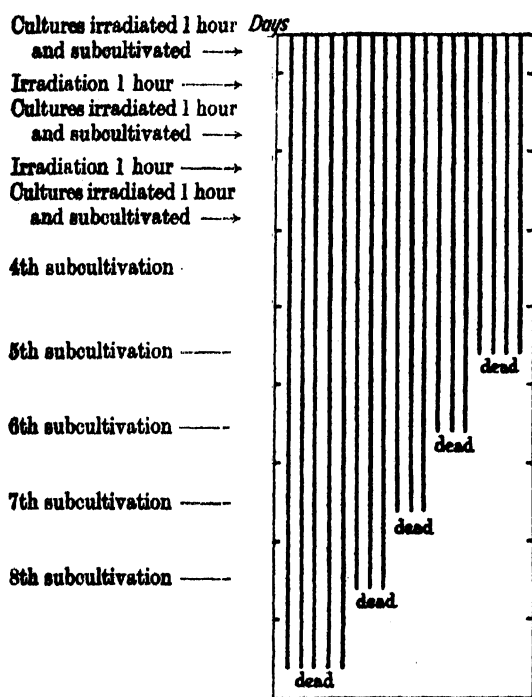


CHART 2.—Showing Survival Periods after Spaced Irradiation of 1 hour daily on six successive days. Cultures of Group I. Each vertical line represents life history of one culture.

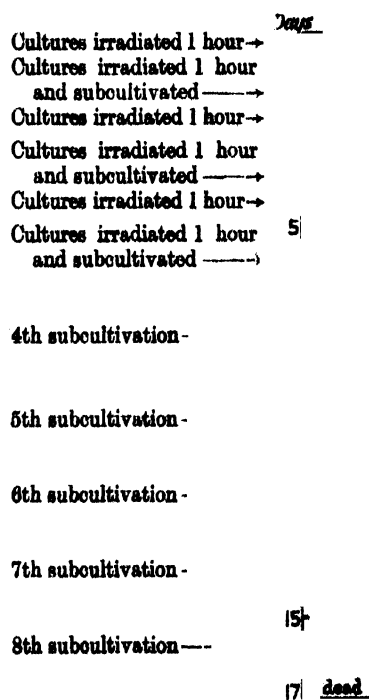


CHART 3.—Showing Survival Periods after Spaced Irradiation of 1 hour daily on six successive days. Cultures of Group II. Each vertical line represents life history of one culture.

Group II.—Cultures were irradiated as in Group I, but subcultivated immediately after the second, fourth and sixth irradiation (see Chart 3).

Four special control cultures were grown in plasma and extract which had been irradiated in separate tubes before use.

Experiments and Results.

(a) *Continuous Irradiation for 6 Hours.*—The appearance of cultures irradiated for 6 hours with 300 mg. radium have been described in detail in a previous paper (Spear, 1930). It was then shown that the average survival of six cultures after irradiation was 6.3 subcultivations.

In the present experiments, 12 additional cultures survived for an average of 6.0 subcultivations after exposure. The average survival period for the total 18 cultures was 6.1 subcultivations.

These results are summarised in Table I and expressed diagrammatically in Chart 1.

(b) *Spaced Irradiation of 1 Hour each Day for Six Successive Days.*—Group I.—The average survival period for 14 cultures was 6.4 subcultivations. Group II.—The average survival period for 6 cultures was 5.2 subcultivations.

The average survival of the total 20 cultures of Groups I and II was 6.0 subcultivations. These results are summarised in Table I and expressed diagrammatically in Charts 2 and 3.

Table I.

	Number of subcultivations before death.	Average.
Continuous irradiation (18 cultures).....	8.8.8.8.8*. 7*.7.7. 6*.6*.6*.5*.5 5.4 4.4.4	6.1
Spaced irradiation (20 cultures)	8† 8 7 7 7 7 7 7 6 6 6 6 5 5 5 5† 5† 5† 4† 4†	6.0

* Cultures included from previous work.

† Group II cultures.

Description of Control Cultures.

A few hours after subcultivation, cells wander out from the tissue into the medium by amoeboid movement until the fragment is surrounded by a zone of cells. This zone consists of evenly distributed feather-like cells which arrange themselves in columns, radiating from the original tissue fragment, like petals of a daisy. Cell division is frequent in this zone after 12 hours' growth, and the number of mitotic figures increases fairly rapidly up to about the 20th hour; thereafter it remains practically constant in a given culture until about the 40th hour of incubation. The number then diminishes. The culture is subcultivated after 48 hours' incubation.

Description of Cultures after Exposures to Spaced Irradiation.

Exposure to radium for 1 hour does not produce a very marked effect on cultures which are subcultivated immediately (Group I, p. 192). After a few hours cells begin to wander out from the explant and mitosis is seen within 24 hours. When a second irradiation of 1 hour is now given and the culture incubated unchanged, the character of the zone of growth is practically unaltered. After the third irradiation (followed by subcultivation) cells wander out from the explant and form a narrow compact mass of tissue with few mitotic figures. Some of the cells in the vegetative state are enlarged and their outlines difficult to see. The fourth irradiation causes little alteration in the appearance of the culture. After the fifth irradiation (and subcultivation) degenerative changes are marked, while cultures subcultivated 24 hours after the sixth irradiation show a very "straggling" outgrowth which appears as short irregular lines of cells well separated from one another. Some cells lie quite isolated; many are much enlarged and none have yet been seen in mitosis.

After each subsequent subcultivation outwandering is markedly diminished. It ceases altogether, as a rule, about 12 days after the first irradiation.

Those cultures which were subcultivated after the second, fourth and sixth irradiations (Group II, p. 192) showed in general the same appearance, but as a group they degenerated slightly more rapidly than the specimens of Group I. Two survived only four subcultivations, three survived five.

The four special cultures which were grown in medium that had been irradiated were indistinguishable from the controls grown in ordinary medium.

Discussion.

An examination of the effects of spaced and continuous radiation obtained by different workers gives apparently conflicting results.

Ancel and Vintemberger (1928) found that the biological effect on eggs of *Rana fusca* was diminished by giving a split dose of X-radiation, and Ancel (1928) found a similar result after the irradiation of lentil roots. On the other hand, Schinz and Slotopolsky (1926) found an enhanced effect on the testicle of the rabbit when a split dose of X-radiation was used. The same result was obtained by Regaud and Ferroux (1927), who showed, however, that a split dose decreased the efficacy of the irradiation when the skin of the same animal was exposed to X-rays.

Alberti and Politzer (1924), working on amphibian larvae, found that the efficacy of spaced radiation varies when the interval between the irradiations

is altered. By choosing a suitable interval they were able to produce an effect corresponding to an irradiation two or three times the sum of the separate doses.

The results of my experiments show that cultures irradiated for 6 hours continuously survive on the average for the same period before death as cultures irradiated for 1 hour daily on six successive days, provided that the subcultivations are counted from the *beginning* of the spaced irradiations. If the survival period is measured from the *end* of the irradiations, cultures are seen to degenerate much more rapidly after the last of the spaced irradiations than after the continuous irradiation owing to the fact that the tissue irradiated in the former case is already much damaged from its five previous exposures.

Spaced radiation thus appears to have a cumulative action in the cells of the original explant, and under the conditions of this experiment it is immaterial whether the dose of radiation is administered all at once or in equal fractional doses, at 24-hourly intervals.

Summary.

Exposure of tissue cultures *in vitro* to gamma rays from 300 mg. (with 0.5 platinum filter) at 0.5 cm. distance for 6 hours caused a delayed lethal effect after an average of 6.1 subcultivations.

Exposure of tissue cultures to the same radium for 1 hour daily on six successive days caused a delayed lethal effect after an average of 6.0 subcultivations measuring from the beginning of the spaced irradiations.

Conclusions.

Under the conditions of the experiment it is immaterial whether an irradiation is administered in one dose of 6 hours or in six fractional doses of 60 minutes each at 24-hourly intervals.

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The Reaction of the Dental Pulp to Peripheral Injury of the Dentine.

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[PLATES 9-14.]

I. Introduction.

A series of experiments by the author (1927) on the dentine of monkeys and dogs, extending from 1924 to the present time, has shown that there is a free fluid path along the dentinal tubules in a space between the fibril of Tomes and the wall of the tubule.

It has also been demonstrated (Fish, 1928) by diffusion experiments that a peripheral injury to the dentine cause stasis of the contents of the injured tubules and that the tract of tubules involved becomes cut off from fluid communication with the pulp by a deposit of secondary dentine. Not only does this tract of tubules become cut off from the pulp, but also by irritating the neighbouring tubules it causes them to deposit lime salts in the communicating branches so that it is ultimately cut off from the surrounding dentine, and is in this way completely isolated physiologically from the rest of the tooth and must be regarded as a dead tract.

These observations have been corroborated by Boedecker (1930), who devised a series of modified experiments to test their accuracy.

The object of the present investigation is to discover the nature of the reactions in the pulp which lead to the isolation of such an injured tract of tubules.

The method by which these reactions were investigated consisted in cutting cavities in the sound dentine of monkeys and dogs, either filling the cavities with cement or leaving them open to the mouth, and at the end of 2, 4 or 12 weeks killing the animal and cutting serial sections of the dentine and pulp *in situ*.

Technique of Experiment.—A dog or monkey is anaesthetised with chloroform and ether and a shallow cavity just entering the dentine is cut in each

of the front teeth with dental burs. It is necessary to keep the bur wet to avoid producing heat which might in itself injure the pulp. The cavity is undercut slightly and filled with copper oxyphosphate cement, or left open to the mouth as the case may be. The pre-determined time is allowed to elapse, whereupon the animal is killed and the teeth are cut out. Each tooth must have the end of the root cut off to expose the pulp to the reagents used in fixing, decalcifying and embedding. The butyl alcohol method is employed and serial sections are prepared and stained with hæmatoxylin and eosin or Van Gieson.

The disadvantage of this method of investigation is that the experimental injuries inflicted upon the dentine may be either less severe or more severe, according to the technique used, than are the natural lesions such as attrition or caries, which occur in human teeth.

If a small experimental cavity is cut in healthy dentine and touched with concentrated carbolic acid following the usual clinical practice, and then sealed hermetically under aseptic conditions with copper oxyphosphate cement, the dentinal tubules, although injured, remain uncontaminated. Nevertheless, having been injured, their contents generally die, but since the tubules are not open to mouth contamination the odontoblasts themselves are not seriously affected, so that having sealed off the dead tract they form a highly tubular type of secondary dentine. It happens occasionally that, provided stagnation is prevented in the injured tubules by intercommunication between their lateral branches, or other means, they remain alive at least for a time and no appreciable reaction at all is observed in the odontoblast layer even after several weeks.

On the other hand, in the case of natural lesions, the tubules are not opened up all at one time, but gradually, one after another. Moreover, each becomes contaminated by mouth fluids and bacteria as soon as it is opened. The result is always death of the tubules together with most of the odontoblasts, and an impermeable barrier of more or less hyaline secondary dentine is laid down at the pulp margin.

If an attempt is made to simulate these natural lesions by cutting an experimental cavity and leaving it unfilled, a very serious discrepancy again exists between the natural and the experimental lesion in that the former is gradual in its development, while the latter lays open a large number of tubules to the mouth fluids at one time.

The effect of these fluids diffusing into the pulp along so many tubules at once may be much more severe than any reaction observed under early caries

Nevertheless, an examination of the serial sections of 177 teeth in which cavities were cut experimentally, and were in some cases filled up and in others left open to the mouth, has shown a series of reactions to the injury which vary in severity, and many of which tend to an end result similar to that observed under the more gradual natural lesions.

An approximate classification of these reactions according to their degree of severity, gives the following figures :—

Number of teeth operated upon	177
Number of these showing no reaction in the pulp	20
Number of these showing changes in the odontoblast layer, and, where sufficient time was allowed to elapse, a deposit of secondary dentine	157

Of the latter group, 25 showed in addition marked round-celled infiltration of the pulp under the injured tract of dentine and 5 showed actual localised granulomata or focal abscesses of the pulp, probably due to infection from the mouth along the injured tubules or from the blood stream.

These figures are probably very largely influenced by variations in technique, since in this preliminary investigation the extent and depth of the lesion in the dentine has not been standardised. The temperature at which the cavity was cut and the thickness of the dentine in the tooth operated upon may also have some bearing on the pulp reactions. The figures, however, serve to show that the changes in the odontoblast layer and the deposition of secondary dentine about to be described are the typical reactions to peripheral injuries of the dentine. An attempt is being made to control the factors involved in the experimental production of the lesion, and a further report will be based on the results.

The changes which occur in the tooth may be considered in three groups :—

- (a) The production of secondary dentine.
- (b) Changes in the odontoblasts associated with the injured tubules.
- (c) General changes in the pulp.

II. *Secondary Dentine Production.*

The phenomenon of secondary dentine production exhibits two distinct phases :—

- (1) Sealing up the injured primary tubules with calcium salts, which constitute a barrier between primary and secondary dentine.

- (2) Formation of the secondary dentine proper, which may be tubular, modified tubular or hyaline.

The reaction of barrier formation and secondary dentine production is always strictly confined to the pulp surface of the injured tract of tubules. It never overlaps the uninjured tubules, and may therefore be regarded as a specific reaction to injury of the dentinal tubules (fig. 1, Plate 9).

The first phase of barrier formation between primary and secondary dentine involves the deposition of calcium salts in the central ends of the injured tubules.

This deposit may be seen at A in fig. 2 (Plate 9)* which is a photomicrograph of a dog's tooth only 2 weeks after the production of the peripheral lesion. It would appear that by cutting across the distal end of the tubule fluid movements therein have been arrested and coagulation has set in and extended as far as the pulp end of the tubule. The ingress of mouth fluids, or even this coagulum itself, then appears to have acted as an irritant to the pulp, which reacted immediately by laying down lime salts at the point of irritation.

Not only are calcium salts deposited some little distance up each tubule, but they continue to be deposited until a considerable layer ("B," fig. 2) has been produced, completing the barrier, whereupon secondary dentine proper ("S," fig. 2) begins to form. Figs. 3, 4, 6, 7B, 7C, 7D, 9 and 12 (Plates 9-14) also show this barrier, the occluding calcium salts in the tubules appearing almost black when stained with hæmatoxylin.

In fig. 3 the secondary dentine has been slightly torn away from the primary in preparing the section and the deposit of calcium in the ends of the primary tubules, together with the further deposit (B), acting as an extra seal is evident. The secondary tubules start quite independently of those in the primary dentine.

Sometimes this first barrier fails to protect the secondary dentine from irritation and apparently leaks at some point. In these cases the secondary dentine already formed shares the fate of the contaminated primary dentine and is in turn sealed off by a secondary barrier (B, fig. 4) and a further deposit of secondary dentine is laid down.

This duplication of barriers is common in human teeth under natural lesions of the dentine and is the normal reaction when caries, having destroyed the primary dentine, penetrates the barrier and infects the secondary dentine (Fish, 1929).

* The teeth figured in the illustrations, except fig. 5, have all been decalcified sufficiently to permit their being cut into sections on the microtome, but enough calcium salts remain to show up clearly when stained with hæmatoxylin.

The physiological efficiency of this barrier can be best observed in ground sections (Fish, 1928) where its high calcium content renders it translucent and emphasises the discontinuity between the primary and secondary tubules (fig. 5, Plate 10).

When an efficient barrier has been formed, the pulp begins to lay down one of the recognised types of secondary dentine. The number of tubules in this secondary dentine depends solely on the number of odontoblasts which survive the initial injury. If most or all of them are destroyed, the secondary dentine is hyaline in type and contains practically no tubules. This is common under natural lesions in human teeth, but the most usual type of secondary dentine under experimental injuries is a modified tubular type in which there are perhaps one-fifth of the usual number of tubules, four-fifths of the odontoblasts having disappeared. When, however, almost all the odontoblasts survive, a highly tubular type of secondary dentine is formed which looks exactly like the primary and is only recognised by observing the presence of the barrier. If a specimen showing this highly tubular type of secondary dentine is decalcified very thoroughly, the calcific barrier (B, fig. 2) will disappear and the tubules of the secondary dentine almost appear continuous with those of the primary, though there is always a twist or kink to indicate the site of the plug of calcium salts which has disappeared.

Fig. 6, like fig. 4, is an example of the modified tubular type. The barrier is clearly shown plugging the dead tubules and the associated reduction in number of both the tubules of the secondary dentine and also of the odontoblasts is evident.

III. *Changes in the Odontoblasts.*

Reference has already been made to the fact that the pulp reactions to superficial injury of the dentine vary in severity. The changes which take place in the odontoblast layer are confined to that part of it which lies immediately under the injured tract of tubules. The end result of this change is death of a proportion of the odontoblasts and the production of secondary dentine either by the survivors or by other cells in the neighbourhood. The secondary dentine acts as a barrier and protects the pulp from further injury by the dead tract of primary dentine.

Typical examples of these reactions under shallow experimental cavities in the teeth of monkeys are shown in fig. 7B, 7C and 7D. In each case four weeks had been allowed to elapse between cutting the cavities and killing the animal.

Fig. 7B shows the least severe reaction. The injured tubules are blocked at B and a small deposit of secondary dentine (S) has formed. The onto-

blasts are distorted and irregular in shape. They have lost their fibrils and some are degenerating, but the majority show regenerative changes with cytoplasmic processes stretching out in all directions, towards the neighbouring cells as though the odontoblasts were taking up their new orientation. They are greatly reduced in number, many having died and disappeared as a result of the injury.

Fig. 7c shows a more severe disturbance. The odontoblasts are still further reduced in number, and show the same degeneration followed by regenerative changes. Small vacuoles appear between them which are presumably due to an exudate lymph. Numerous new capillaries are seen growing down into the damaged area to assist in the process of repair. The result of this more severe disturbance is that no secondary dentine has as yet formed, and indeed the normal odontogenetic zone (Og, fig. 7A) of the primary dentine has disappeared. Lime salts are, however, being deposited in the ends of the injured tubules and some collagen fibres (D) are seen preceding secondary dentine formation.

Fig. 7d shows the complete disorganisation of the odontoblast region under a similar peripheral lesion of the dentine. The odontoblasts have almost entirely disappeared, together with the odontogenetic zone of the dentine, and are replaced by a mass of red blood cells and round cells with occasional polymorphonuclear leucocytes. The dead primary tubules are blocked with lime salts, but there is no attempt as yet to lay down secondary dentine.

In each case these disturbances in the pulp are extremely local and are confined to the tissues immediately under the injured tubules, so that it would appear that a peripheral injury to the dentine not only results in the death of the injured tubules but also causes a reaction in the pulp resulting in the local destruction of some of the tissue elements followed by the usual phenomena of repair modified to meet the peculiar circumstances of the part.

The end result of all these changes is a boss of secondary dentine such as that in fig. 1 with a layer of normal odontoblasts adjoining it, surrounded by normal pulp tissue but with a reduction in the number of odontoblasts at the site of the lesion depending upon the severity of the original reaction and determining the number of tubules in the secondary dentine.

A feature of some of these reactions to peripheral injury of the dentine is the appearance of large oval cells twice the diameter of the normal odontoblast showing a well-defined nucleolus in a delicate tracery of chromatin (fig. 8A).

These cells are exactly like certain connective tissue cells normally present in the pulp, except that they tend to stain somewhat more definitely in their new situation. In a fully-developed tooth such cells are only found behind

the odontoblast layer in the general pulp tissue, but in some cases of injury to the dentine, especially when all or nearly all of the odontoblasts have disappeared, these cells come to lie at the developing edge of the secondary dentine, each with its long axis parallel to it."

Similar cells are found amongst the odontoblasts of developing teeth (fig. 8b) and their appearance at the site of development of secondary dentine suggests that they may have some definite rôle in the production of calcified tissue by the pulp.

IV. *General Changes in the Pulp.*

Frequently the only apparent reaction to peripheral injury of the dentine is the production of secondary dentine with its associated changes in the odontoblast layer, but in 40 out of the first 85 teeth in which experimental cavities were cut, hæmorrhages appeared in the pulp. These were sometimes slight, but often quite severe, and while they generally occurred in the vicinity of the damaged tract of dentine, they could also be observed at remote points in the pulp (fig. 9, Plate 13).

When the animal is killed and the tooth sectioned on the day following operation, the hæmorrhage is already invaded by polymorphonuclear leucocytes (fig. 10, Plate 13) showing that it must have occurred at the time of operation 18 hours before, but sections of teeth which were extracted some weeks after operation show that the hæmorrhages are not absorbed and that no phagocytosis is taking place. The red cells are distorted, but no pigment is to be seen and no polymorphonuclear leucocytes.

On the other hand, the hæmorrhages also clearly occurred at the time of operation some weeks earlier, because they are to some extent invaded by lymphocytes, although not enough to suggest that they are a serious source of irritation to the pulp tissue (fig. 11, Plate 13)

Control teeth in which no cavity is cut do not show these hæmorrhages, although in normal teeth a minute escape of red cells from the vessels has been observed occasionally, to which there is no reaction, suggesting that the pulp may resemble reticulum and have hæmothigmic properties (Tait, 1927).

It is not entirely clear why a shallow peripheral injury to the dentine should cause hæmorrhages of the pulp, but it is suggested that they are the result of mechanical decompression of the pulp following the opening of the tubules. It has been found that the more extensive the cavity is in the dentine the more certain the hæmorrhages are to occur. A peculiarity of the blood vessels of the pulp is that even the largest ones corresponding in size to arterioles have only an endothelial coat. There is no muscular or fibrous wall. This permits

impulses to be conveyed to the dentinal tubules and promotes the movements of tissue fluid in the pulp and dentine, and since the pulp chamber is a closed cavity except for the minute apical canals there is normally little risk of rupture. At the same time the very delicacy of these vessel walls which permits the transmission of impulses to the tubules would render the vessels liable to burst if the hydrostatic equilibrium of the dentine and pulp were disturbed by the sudden opening of any large number of the tubules involving an immediate decompression, and the rupture would the more readily occur if there were a reactive dilation of the vessels to injury as occurs elsewhere in the body.

Another less common condition to be observed under experimental cavities of the dentine is the invasion of the odontoblast layer by capillaries which come to lie between the injured dentine and the odontoblasts (*cf.* figs. 7c and 8A). In this position the lymph which exudes from the capillaries will not only irrigate the surface of the injured dentine but will move past and between the displaced odontoblasts, preserving their vitality. Very commonly this is associated with a round-celled invasion and the result is a minute mass of granulation tissue.

Orban (1928) refers to the presence of capillaries in the normal odontoblast layer, but while they do occur it is very clear that in the cases where they have been observed in the odontoblast layer after injury to the associated dentine, they are a new growth of capillaries. They are localised to the area of the lesion, are unusually numerous, and are distributed evenly along the margin of the injured dentine.

After a few weeks these capillaries recede and the surviving odontoblasts form a reduced odontoblast layer and lay down tubules in the secondary dentine.

Reference has been made to a more severe type of reaction which is observed in about 3 per cent. of cases, where the odontoblasts disappear entirely and are replaced by a solid mass of round cells into which new capillaries are growing. Two such lesions resembling granulomata are shown in the same tooth (figs. 12 and 13), and in this case the larger one is breaking down in the centre and the round cells at this point surround a collection of polymorphonuclear leucocytes, though the rest of the mass consists of lymphocytes only. It would, therefore, be more correct to describe this lesion as a focal abscess since there is very probably infection present at its centre.

This reaction occurred under a 10 weeks' old injury to the surface of the dentine in a monkey's tooth. Bosses of secondary dentine alternate with the

granulomata and indicate a difference in the severity of the reaction at different points along the pulp margin under the injured tract of tubules.

The edge of the dentine itself opposite these granulomatous masses appears to be undergoing a species of absorption. This is not the usual type of absorption by means of osteoclasts such as occurs in ordinary cases of absorption of the dentine by the pulp. Nevertheless it is a constant feature of these small experimental granulomata of the pulp and also of similar lesions occasionally observed in the pulp under natural erosion of the necks of human teeth.

These granulomata at the edge of the dentine are completely localised and the rest of the pulp remains quite normal.

Finally, it has been observed that a peripheral injury to the dentine may produce so severe a reaction that the whole pulp is killed without it having been exposed. In these cases, after a week or two, only a mass of debris is observed in the pulp canal.

V. Discussion.

The observations just recorded indicate that the reaction to peripheral injury of the dentine is very similar to the ordinary inflammatory reaction which follows a superficial wound of the soft tissues.

There are two differences. One is that the reaction in the case of the tooth takes place in the pulp and not at the actual site of the injury, and therefore the reaction is somewhat delayed and modified by the interposition of the dentinal tubules, along which any irritating foreign body must travel before it can injure the pulp. If such irritants are prevented from entering the wound in the dentine the reaction in the pulp may be delayed, though generally degeneration of the odontoblasts occurs at once and is followed by regeneration of a proportion of them and secondary dentine formation. This represents simply the reaction of the pulp shutting off an injured tubule, the contents of which, though dead, remain aseptic.

On the other hand, if irritating fluids do enter an injured tubule they may cause serious damage to the pulp, and the difference between the two reactions is as marked as the difference between a wound healing by first intention and one which heals by granulation (*cp.* figs. 7B and 7D).

Hitherto, the only definite reaction to injury of the dentine which has been recorded is the occurrence of the translucent zone under caries, but this is not a constant phenomenon and can only be observed in the very early stages of the disease when the dentine is merely irritated by the proximity of enamel caries or by exposure at the neck of the tooth.

The translucent zone is a hypercalcification of the dentine due to a deposit

of lime salts in the tubules and it can, therefore, only be produced while the tubules are intact and while they are still receiving a supply of tissue fluid. Once they are invaded by the disease, toxic material diffuses into them and their contents are destroyed. Any hypercalcification which may have taken place already will remain until that part of the dentine is entirely destroyed by caries, but no further vital reaction can take place in it.

The second important difference arises from the fact that one of the functions of the pulp is to lay down calcium salts and therefore, the end result of an inflammatory reaction in it tends to be the production of a calcified scar, that is secondary dentine, rather than the production of fibrous tissue. Nevertheless, the secondary dentine may be delayed if the injury is very severe and a chronic granuloma may take its place, at any rate, for some months.

A parallel may also be found for the hæmorrhages which take place in the pulp when a large number of dentinal tubules are opened, thereby modifying the hydrostatic equilibrium of the pulp. Similar hæmorrhages occur in the retina when the intra-ocular tension is suddenly lowered by operation, especially in cases of glaucoma.

It would appear from these investigations that, as pointed out by Sir Arthur Keith, nature makes no attempt to repair a carious cavity in the dentine, but it is clear that an elaborate and efficient barrier is constructed to protect the living tissue of the pulp from contamination by the dead tubules when any part of the dentine is destroyed.

Summary.

(1) In earlier experiments it has been demonstrated that when dentinal tubules have been opened to the mouth they die, forming a dead tract physiologically cut off from the pulp by secondary dentine and from the surrounding dentine by hypercalcification in the uninjured neighbouring tubules.

(2) The secondary dentine always exactly corresponds in its distribution to the tract of tubules which has been injured, and is a specific reaction to peripheral injury of the dentine (fig. 1).

(3) The barrier between primary and secondary dentine consists of calcium salts sealing the ends of the injured tubules of the primary dentine (figs. 2, 3, 4, 5, 6, 7, 9 and 12).

(4) The following reactions are described as occurring in the pulp after an experimental injury to the periphery of the dentine.

(a) The odontoblasts associated with the injured tubules degenerate and many of them die and disappear. The remainder show regenerative

changes and return to normal, laying down the tubules in the secondary dentine (figs. 6, 7 and 9).

- (b) The number of tubules in the secondary dentine depends on the number of odontoblasts which survive the injury. If none survive, the secondary dentine is hyaline (figs. 6 and 8A).
- (c) An embryonic type of cell often appears after a few weeks at the developing edge of secondary dentine. It may be a connective tissue cell concerned in the production of secondary dentine (fig. 8).
- (d) Hæmorrhages often occur in the pulp and are probably due to decompression. The red cells are not quickly absorbed, but undergo a slight round-celled infiltration (figs. 1, 8A, 9, 10, 11).
- (e) If the injury is severe, round-celled infiltration and invasion of the odontoblast layer by capillaries takes place (figs. 7 and 8).
- (f) In more severe injuries still, small marginal granulomata of the pulp appear in contact with the injured tubules instead of the normal secondary dentine formation (figs. 12 and 13).
- (g) Death of the pulp may follow the most severe peripheral injuries of the dentine.

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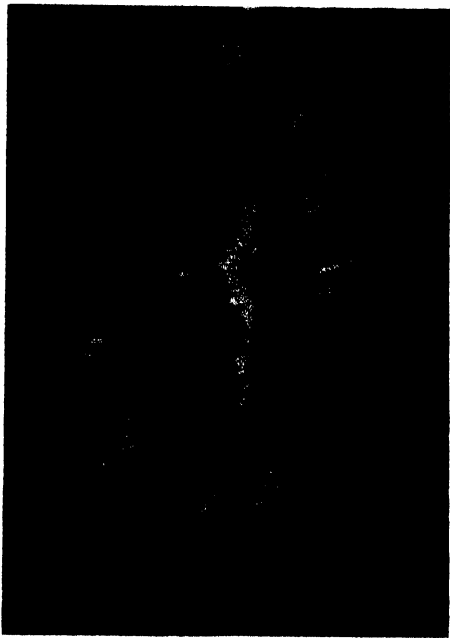
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EXPLANATION OF PLATES.

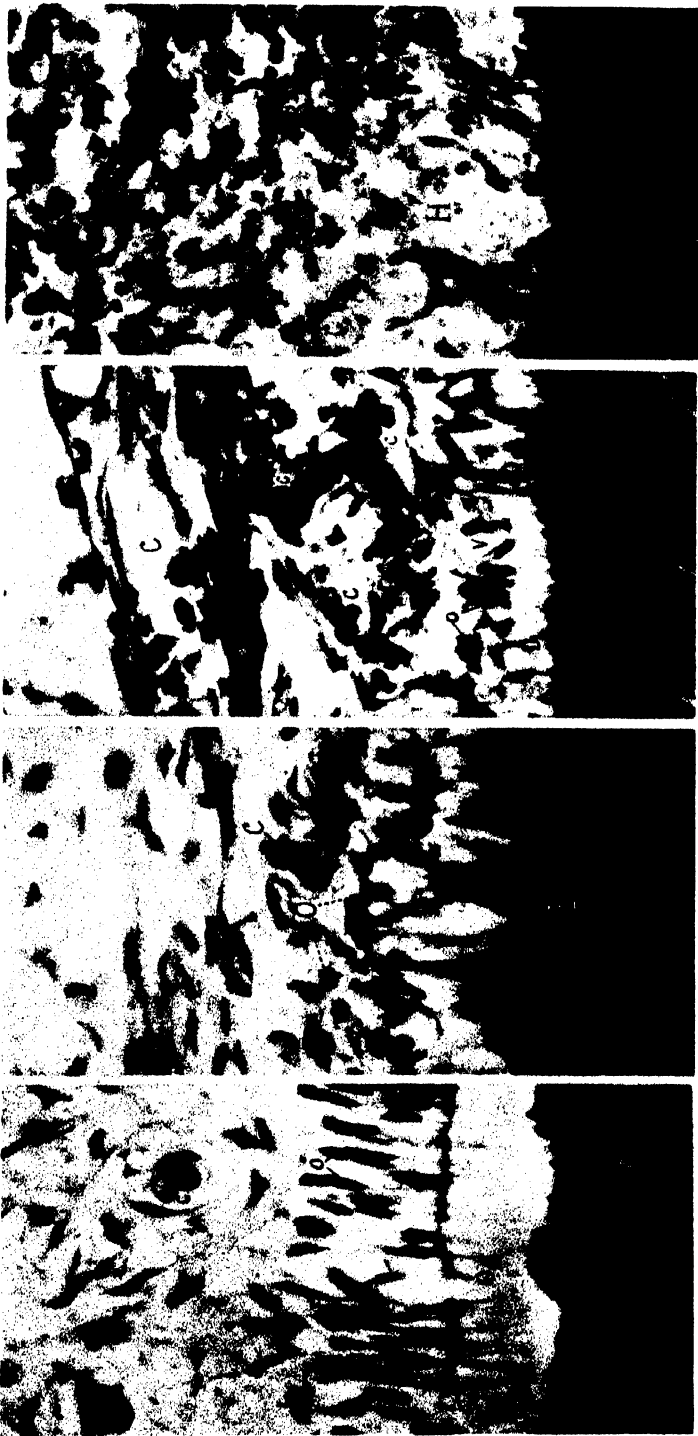
PLATE 9.

FIG. 1.—Photograph of tooth of monkey $\times 32$. Experimental cavity (C) cut 12 weeks before death. The secondary dentine (S) separated from the primary by the barrier (B) exactly corresponds to the tract of tubules injured at C. There are hæmorrhages of the pulp at H.

FIG. 2.—Photomicrograph of tooth of dog 3 weeks after cutting the experimental cavity $\times 544$. The deposit of lime salts (B) is observed to extend a short distance up the injured tubules (A) and forms a barrier between the highly tubular secondary dentine (S) which is beginning to form and the injured primary dentine.







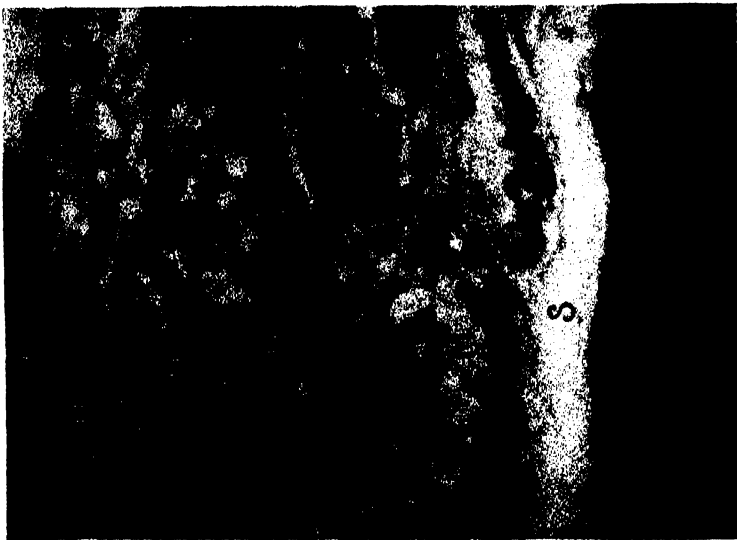
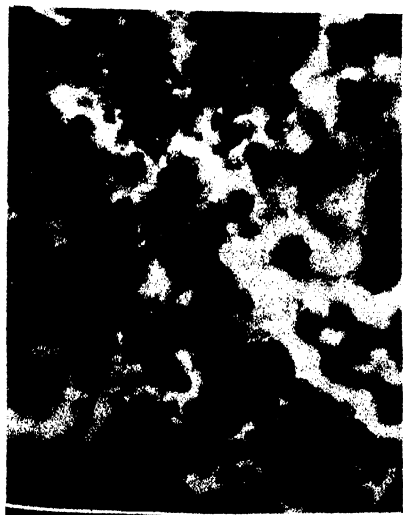
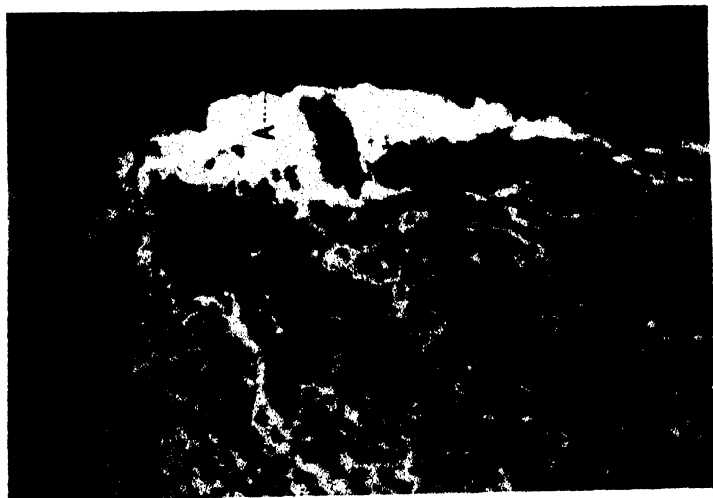




FIG. 9.





FIG

FIG. 3.—Photomicrograph of monkey's tooth 12 weeks after cutting an experimental cavity $\times 544$, showing the injured primary tubules sealed with lime salts at A, the barrier (B) between primary and secondary dentine and the origin of the secondary tubules (T).

FIG. 4.—Photomicrograph of tooth of monkey 12 weeks after cutting the experimental cavity $\times 76$. The barrier is seen at A sealing off the primary tubules and a second barrier appears at B.

PLATE 10.

FIG. 5.—Photomicrograph $\times 360$ of a ground section of a monkey's tooth 8 weeks after cutting an experimental cavity, showing the translucent barrier of calcium salts (B) and the origin of the secondary tubules (S).

FIG. 6.—Photomicrograph of tooth of monkey 4 weeks after cutting the experimental cavity $\times 544$. The barrier of lime salts (E) extends into the injured tubules (A). A deposit of modified tubular secondary dentine has formed. The odontoblasts are much reduced in number and embryonic in type. There is a round-celled infiltration (R) of the adjoining pulp tissue.

PLATE 11.

FIG. 7A.—Normal odontoblast layer of monkey for control $\times 544$. (O) odontoblasts, (C) capillaries, (Og) odontogenetic zone.

FIG. 7B.—Photomicrograph of a monkey's tooth 4 weeks after carefully cutting a very shallow experimental cavity and sealing it with cement $\times 544$. The odontoblasts (O) are considerably reduced in number, and have degenerated. Some are regenerating and their cytoplasm is drawn out into fine processes reaching out towards neighbouring cells. Some secondary dentine (S) has formed and a capillary is seen at C.

FIG. 7C.—Photomicrograph of a monkey's tooth 4 weeks after cutting an experimental cavity $\times 544$, showing more serious degeneration of the few remaining odontoblasts (O). Some collagen fibres (D) indicate the commencement of secondary dentine formation. Vacuolation (V) may still be seen and the capillaries (C) and (C) have invaded the odontoblast layer. The odontogenetic zone has disappeared.

FIG. 7D.—Photomicrograph of a monkey's tooth 4 weeks after cutting an experimental cavity $\times 544$. The odontoblasts are entirely replaced by degenerating red blood corpuscles (H) following a hæmorrhage and a round-celled infiltration (R). The odontogenetic zone has disappeared and the injured tubules are sealed with calcium salts. There is no secondary dentine formed as yet.

PLATE 12.

FIG. 8A.—Photomicrograph of a monkey's tooth 8 weeks after cutting an experimental cavity $\times 544$. Showing large oval mononucleolated cells (M) at the edge of the dentine. There is a large hæmorrhage of the pulp (H) and capillaries (C) may be seen in the vicinity. This reaction is exactly comparable in severity with that shown in fig. 7D, in that the odontoblasts are replaced by red cells and round-celled infiltration, but in the extra 4 weeks which have elapsed, the cells (M) have appeared and a layer of hyalin secondary dentine (S) has been laid down.

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FIG. 8a.—Photomicrograph $\times 544$ of developing tooth of 2 days' puppy. Similar oval mononucleolated cells to those in fig. 8A may be seen at M between the odontoblasts (O).

PLATE 13.

FIG. 9.—Higher magnification of tooth shown in fig. 1 (later section in the series) $\times 110$, showing hæmorrhages (H and H) invaded by round cells (R). The reduction in the number of odontoblasts and consequently also of the tubules in the secondary dentine is very marked.

FIG. 10.—Photomicrograph $\times 544$ of hæmorrhage in the pulp of a dog's tooth 18 hours after cutting the cavity. The hæmorrhage is invaded by polymorphonuclear leucocytes only.

FIG. 11.—Photomicrograph $\times 544$ (higher magnification of fig. 9) showing hæmorrhages after 12 weeks invaded only by lymphocytes. There appears to be no phagocytosis taking place.

PLATE 14.

FIG. 12.—Photomicrograph of monkey's tooth 10 weeks after cutting experimental cavity $\times 70$. A barrier has been laid down at B and bosses of secondary dentine (S and S1) have formed. Between and beyond these bosses are granulomata (G) and erosion of the dentine has occurred at A.

FIG. 13.—Higher magnification of fig. 12 $\times 290$. Part of one of the granulomata (G) showing degeneration of the round cells and the presence of leucocytes. Capillaries are seen at (C) and erosion of the edge of the dead tract of dentine has occurred at (A).

Experiments on Growth and Inhibition. I.—The Increase of Inhibition with Distance.

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Introduction.

Experiments were recently reported showing that, in young seedlings of *Pisum sativum*, the complete inhibiting effect which the shoot exerts upon its axillary buds comes entirely or almost entirely from three or four of its developing leaves acting together (6). A single developing leaf was found usually to inhibit only partially—that is to say, sufficiently to delay the growth of an axillary bud below it, but not to check it completely. The strength of this partial inhibiting effect was measured by the retardation of the outgrowth of the axillary buds of the first or lowest leaf, as compared with their growth in completely defoliated controls.

Comparisons were further made of the inhibiting effects of single young leaves of equal sizes near the apex in seedlings of different ages and heights, and it was found that in very young short seedlings the inhibiting effect was very slight or inappreciable, although in seedlings of a height of about 30 mm. or more (but still possessing well filled cotyledons) the effect was strong.

This quite unexpected result seemed of great interest, since it suggested that, unlikely though it may at first appear, the inhibiting effect exerted by the young leaves upon an axillary bud may actually be increased by a longer stretch of stem intervening between them. None the less, as was pointed out at the time, the results did not by any means establish this. For the taller seedlings were also several days older, so that, besides possessing a greater intervening length of stem, they must have differed from the short ones in many other ways also. It may therefore have been on account of some of these other differences that their young leaves inhibited more strongly.

The next step required, therefore, was clearly to determine whether in seedlings of equal age inhibition is stronger when the intervening length of stem is greater, other things being equal. But the provision "other things being equal" is, here, one with which it is difficult to comply strictly. The following

experiment, however, comes near to being what is required. Its possible source of error will be discussed afterwards.

Experiments.

Experiment 1.—Seedlings of *Pisum sativum* (of race Thomas Laxton, as in all the experiments here reported) were grown in the dark, in deep boxes, each containing 25 or 30 seedlings sown at the same time. When their fifth leaves had reached a length of from 5 to 10 mm. operations were performed upon them for the purpose of comparing the inhibiting effect exerted by these leaves upon the axillary buds of the third leaves close below them with their effect upon the more distant axillary buds of the first leaves. The operations were of four kinds, so that four groups of seedlings were formed, A, B, C and D (see fig. 1). In groups A and C the seedlings were decapitated above the

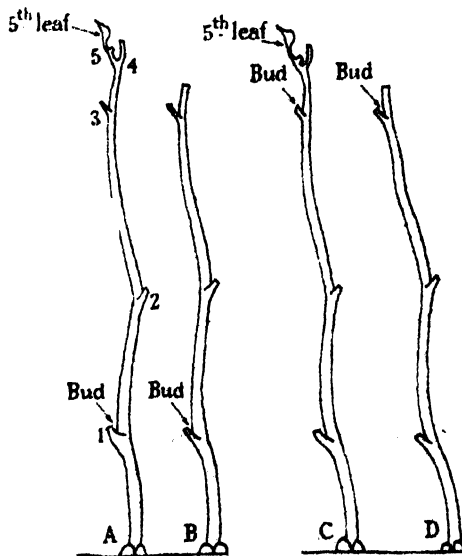


FIG. 1.—Illustrating experiment 1. The arrows indicate the positions of the remaining buds, and of the remaining leaves in A and C. The nodes are numbered in A.

fifth node and in groups B and D above the third node. Also in all the groups they were deprived of their leaves and of the axillary buds of the shoots until only the following leaves and axillary buds remained :—

In A, the fifth leaf and the axillary at the first node ; in B, the axillary at the first node, but no leaves ; in C, the fifth leaf and the axillary at the third node ; in D, the axillary at the third node, but no leaves. (The axillaries of

the cotyledons also remained, but may be disregarded since they scarcely grew at all in any of the experiments here reported.)

The boxes of seedlings were then placed in bright diffuse light in a greenhouse, and after from 4 to 7 days, according to temperature, the axillaries were measured. The ratio of their mean growth in A to that in B served as a measure of the inhibition when the intervening stretch of stem was long and the ratio of the growth in C to that in D measured it when the intervening stem was short. The point was, therefore, to compare these ratios.

The length of stem between the inhibiting leaf and the remaining axillary bud was, in "A" from 70 to 100 mm. at the time of operation, whereas in "C" it was only from 5 to 15 mm., except in one plant in which it was 23 mm. During the experiments these intervening lengths of stem usually grew from 25 to 35 mm. more. The reason for growing the plants in the dark was merely that the difference between the distances in A and C was thereby made much greater. The fifth leaves were from 5 to 10 mm. long at the start, and they grew during the experiments to lengths of from 18 to 38 mm. They were found in preliminary experiments to exert too strong an inhibiting effect for convenient measurement when intact, and they were therefore reduced in size at the time of operation, by the removal of both their stipules and of one of their two pinnae.

From nearly every box of seedlings, some seedlings were selected for each of the four groups but it was not usually convenient to use equal numbers of plants for all four. Since, however, the operations in groups A and C were performed when the fifth leaves had reached approximately the same size, the plants of these groups must have been at practically the same stage of development or physiological age. Care was also taken that the plants of the "control" groups B and D should be at approximately the same stage of development as those of the corresponding groups A and C, and closely similar to them in general vigour and in the initial size of the bud to be measured.

The measurements were usually made after the same times in C and D as in A and B, but sometimes they were made a little later. The reason for this was that the third-leaf axillaries of control group D sometimes grew out slightly more slowly than the first-leaf axillaries of control group B, probably because they were slightly less developed at the start. It was therefore thought best to allow, in each batch of plants, the buds of the control groups B and D to grow just until they reached about a certain convenient mean length (from 4.5 to 5 mm.). As soon as the buds in B reached about this length, their lengths were recorded and also those of the buds in the corresponding experimental group A. Similarly as soon as the D buds reached about this length,

both they and the C buds were measured. The growth of the buds was reckoned by subtracting from their final length an allowance of 0.75 mm. for their length at the start, since in these etiolated seedlings they were fairly uniformly of approximately this length.

The cotyledons remained plump throughout the experiment. Any plants in which they became unhealthy were rejected.

The following results were obtained :—

In group "A" the mean growth of the measured bud, in 24 plants, was 2.07 mm., with $\sigma_m = 0.213$.

In control group "B," in 29 plants, it was 4.22 mm., with $\sigma_m = 0.114$.

In group "C," in 23 plants, it was 3.06 mm., with $\sigma_m = 0.187$.

In control group "D," with 24 plants, it was 3.96 mm., with $\sigma_m = 0.163$.

Let the four means be called M_a, M_b, M_c, M_d . The ratio $M_a : M_b$ is 49 : 100, while the ratio $M_c : M_d$ is 77 : 100. Thus the "A" buds were more strongly retarded than the "C" buds.

For a statistical examination of the results, however, it will be easier to compare the differences $M_b - M_a$ and $M_d - M_c$ instead of the ratios. But in order that these differences might be strictly comparable, it would clearly be necessary that the means in the control groups B and D should be equal. For this reason the measurements were in fact made at such a time as to make the means of these two groups as nearly equal as possible. But even so they differ slightly, M_d being the smaller. Let us then suppose that the buds in groups C and D had been allowed to grow on until their mean growth in D had reached a new value $M_{d'} = M_b$. It can be assumed, without risk of more than very slight error, that the buds of group C would have grown on at a proportional rate,* so that their mean growth would have reached a value $M_{c'} = M_c \times \frac{M_b}{M_d}$. A comparison can therefore be made between the differences $(M_b - M_a)$ and $(M_{d'} - M_{c'})$.

Numerically, $M_{d'} = M_b = 4.22$, and $M_{c'} = 3.06 \times 1.06 = 3.24$.

$$M_{d'} - M_{c'} = 0.98,$$

$$M_b - M_a = 2.15,$$

therefore

$$(M_b - M_a) - (M_{d'} - M_{c'}) = 1.17.$$

The standard deviations of M_b and M_d must also be multiplied by M_b/M_d .

* The same assumption would really be implied if the ratios of the means were compared, since otherwise it would not be justifiable to compare them at a time when M_b and M_d were not quite equal.

Therefore

$$\sigma(M_c) = \sigma(M_b) \times 1.06 \times 0.19,$$

$$\sigma(M_d) = \sigma(M_b) \times 1.06 = 0.173.$$

Then

$$\begin{aligned} \sigma((M_b - M_a) - (M_d - M_c)) \\ = \sqrt{\sigma^2(M_a) + \sigma^2(M_b) + \sigma^2(M_c) + \sigma^2(M_d)} = 0.356. \end{aligned}$$

Therefore the difference between $M_b - M_a$ and $M_d - M_c$ is 3.29 times its own standard deviation and fully significant.

It appears therefore that in the plants of group A, with much greater intervening length of stem, the axillary buds were more strongly inhibited than in those of group B. It will be considered below whether any other interpretation is possible.

In the following earlier experiment (experiment 2) apical buds were used instead of single leaves to provide the partial inhibition. Otherwise the arrangement was the same as in experiment 1, except that, by an oversight, the plants of control group B, with first-leaf axillaries remaining, were decapitated above the first leaves, whereas it would have been more strictly correct to decapitate them above the third leaves, as was done in experiment 1, in order to make them similar in this respect to the plants of control group D. Reasons will, however, be given below for considering that this difference did not affect the results.

The leaves in groups A and C were removed up to the sixth leaves inclusive when these were between 2 and 4.5 mm. in length—usually between 3 and 3.5 mm. Consequently the largest remaining leaves of the apical buds (the seventh) must have been of a mean length of about 1.25 mm., and the axillary buds below grew out, but were still partially inhibited (see (6), p. 350). The seventh leaves were removed in turn if they reached a length of about 3 or 4 mm. before the end of the experiment. Etiolated seedlings were used, as in experiment 1, though sometimes they were grown in light for the first few days, before being put in the dark. After the operations they were placed in diffuse light as in experiment 1. In several of these etiolated seedlings, the young leaves, from the seventh upwards, were found to grow very slowly. This difficulty was met by rejecting all plants in which the seventh leaves failed to reach a length of 2.75 mm. or more during the experiment.

The length of stem between apex and axillary bud at the time of operation varied in A from 70 to 170 mm. and in C from 14 to 42 mm. This great variation in stem length was probably due to the fact that some of the batches

of seedlings were put in the dark at an earlier stage than others. During the experiment the stems usually grew between 25 and 40 mm. more. The cotyledons remained well-filled throughout. The results obtained were as follows :—

In "A" the mean growth of the remaining axillary bud, in 16 plants, was 1.77 mm., with $\sigma_m = 0.171$.

In "B," in 25 plants, it was 4.43 mm., with $\sigma_m = 0.193$.

In "C," in 22 plants, it was 2.95 mm., with $\sigma_m = 0.135$.

In "D," in 26 plants, it was 3.74 mm., with $\sigma_m = 0.230$.

The ratio of the means in A and B is 38.5 : 100, and of those in C and D, 78.9 : 100. Thus again the "A" buds were more strongly retarded than the "C" buds. Indeed the difference between them is greater than in experiment 1.

A calculation made in exactly the same way as in experiment 1 shows that the difference between the retardation of the "A" buds and that of the "C" buds is 4.22 times its own standard deviation and thus fully significant. Again, therefore, inhibition appears to have been stronger in the plants with greater intervening length of stem.

It might, however, be objected that in experiment 2 the plants of control groups B and D were not strictly comparable, since, as already mentioned, in B they were decapitated above the first leaf instead of the third. It might, therefore, be suggested that if they had been decapitated above the third leaf, and so had retained a greater length of mature stem above the axillary buds, the buds might for some reason have grown out less rapidly; and if so, then the difference $M_b - M_a$ would have been diminished.

In order to test this possible objection, the following experiment was performed (experiment 3). Etiolated pea seedlings at about the same stage of development as those of experiment 2 were defoliated, deprived of all axillary buds except those of the first leaves and decapitated either (a) above the first leaves, or (b) above the third leaves. They were then placed in diffuse light. The length of stem that remained above the axillary bud was greater by about 100 mm. in (b) than in (a).

After 5 days, the mean growth of the axillaries in (a), in 16 plants, was 3.72 mm. ± 0.087 , while in (b), in 15 plants, it was 3.78 mm. ± 0.128 . The difference between the means is very small and not nearly significant. There is therefore no evidence that in seedlings decapitated above the third leaf, the axillaries of the first leaves grow out any less rapidly than in those decapitated above

the first leaves, and the objection to experiment 2 considered above gets no support.

The results of experiments 1 and 2 agree therefore with those reported previously in showing that axillary buds are inhibited more strongly when there is a greater length of stem between them and the inhibiting members. And further, in the present experiments this difference cannot have been due to greater age of the plants with greater length of stem, for the plants compared were of the same age. But it may still be objected that, none the less, the axillary buds measured in groups A and B being those of the first leaves, were older by about a week than those of the third leaves measured in C and D; and that the older buds may be for some reason more susceptible to inhibition. This suggestion would not indeed be very plausible and the writer knows of no evidence tending to support it. Moreover, it must be noted that actually the axillaries of groups A and B seem to have been, in a general way, slightly more vigorous than those of C and D. For, as the results show, the axillaries of control group B, with no inhibiting leaf, grew slightly more than did those of control group D, although (as explained above) the mean times after which the axillaries were measured were slightly less in A and B than in C and D. When, therefore, it is considered that in both experiments the buds in group A were much more strongly inhibited than those in group C, it seems extremely unlikely that the results are to be explained as due to the slightly greater age of the A buds. Such an explanation is, however, theoretically possible.

Another way of testing the stem-length effect is to compare the strength of inhibition in seedlings grown in light with that in tall etiolated seedlings. This experiment is open to the objection that the plants compared will differ not only in length of stem, but also in their general physiological condition and in that of their leaves and buds. None the less, since the experiment is simple and obvious, it seemed worth while to carry it out. It was arranged as follows.

Experiment 4.—Pea seedlings of the same race, as before, were grown in light, in the greenhouse, and were operated upon in exactly the same way as the etiolated plants of groups A and B of experiment 1. The two corresponding groups in the present experiment will be called E and F. The fifth leaves were from 6.5 to 10.5 mm. in length at the start of the experiment and from 20.5 to 35.0 mm. at the end. They were therefore of approximately the same size throughout as in experiment 1. But the lengths of stem intervening between the fifth leaves and the first-leaf axillaries were only from 21 to 32 mm. at the start and from 40 to 58 mm. at the end, and were, therefore, much less than in

groups A and B of experiment 1, though considerably greater than in groups C and D of that experiment. The cotyledons remained well filled all the time.

The following results were obtained :—

In "E," the mean growth of the remaining axillary, in 30 plants, was 2.92 mm., with $\sigma_m = 0.180$.

In "F," the mean growth in 30 plants was 4.37 mm., with $\sigma_m = 0.101$.

The ratio $M_e : M_f$ is 66.8 : 100, whereas in experiment 1 the ratio $M_e : M_f$ was 49 : 100. Thus the "E" buds of this experiment were less strongly retarded than the "A" buds of experiment 1.

A calculation made in the same way as in experiments 1 and 2 shows that the difference between the retardation of the "E" buds and that of the "A" buds of experiment 1 is 2.41 times its own standard deviation. There is, therefore, a probability of about 60 to 1 that it is significant, and consequently that in the plants grown in light, with shorter intervening length of stem, the buds were inhibited less strongly than the corresponding buds in the tall etiolated plants of experiment 1.

So far as they go, therefore, the results of experiment 4 support those of experiments 1 and 2. They are, however, as already pointed out, open to the objection that the plants compared differed in other respects besides in their length of stem. Consequently the value of experiment 4 as evidence for the stem-length effect is very much less than that of experiments 1 and 2.

In another experiment (experiment 5) comparisons were made of the intensities with which a developing leaf inhibited several buds at different distances from it in the same plant. Pea seedlings were used which had been decapitated in the epicotyl when very young, so that the axillary buds of the cotyledons had grown out and formed two new shoots. They were grown in diffuse light, and those were selected in which the two shoots were nearly equal or not very unequal. The longer shoots of each plant were decapitated above their fourth leaves, when these were in the stage of rapid development, being from 5.5 to 14 mm. long, and at the same time the shorter shoots were decapitated above their second leaves, which were already mature (see fig. 2). The axillary buds of the two lowest leaves of each shoot were allowed to remain; those on the longer shoots will be called L1 and L2, and those on the shorter shoot S1 and S2. The higher axillary buds of the longer shoots were removed. The object was to note the order in which the four remaining buds would grow out, and hence to discover how the strength of the inhibition coming

from the young fourth leaves would vary at different distances as it travelled down the longer shoots and up the shorter ones. During the first few days, a little inhibition must have come from the third leaves also, which were still growing, but the first and second leaves of both shoots were already full-grown at the start, and it was shown previously (6) that in *Pisum* full-grown leaves do not inhibit. The fourth leaves were deprived of one pinna and both stipules since they were found to inhibit too strongly for convenience when intact.

The figure shows that the distances from the fourth leaves to the buds increased in the order L2, L1, S1, S2, and this was found to be also the order of priority in which the buds most often grew out. In detail, the results were as follows.

The number of plants operated upon was 36, and amongst these L2 grew out first 28 times, equal first with L1 once, and second to L1 seven times.

L1 grew out before S1 35 times, and equally with S1 once. S1 grew out before S2 24 times, and after S2 12 times.

There were no double reversals of the usual order—that is to say that S1 never grew out as soon as L2, nor S2 as soon as L1.

It is of interest to compare also the times taken by the buds to reach some conveniently chosen length, say 2.5 mm. The means of the times taken to reach this length were 6.27 days for L2, 6.72 days for L1, and 9.42 days for S1 or S2, whichever grew the sooner.

Before interpreting these results, it is necessary to compare the rates at which similar buds grow out when no developing leaves are present. Accordingly (experiment 6) 29 other shoots springing from the axils of cotyledons, roughly similar to the longer shoots of experiment 5 and grown under the same conditions, were decapitated above their mature second leaves as controls. Only one of these shoots was left on each plant. Of the two axillary buds remaining on each shoot, bud (1), the lower one, grew out first 14 times, equally with bud (2) 4 times and after bud (2) 11 times.

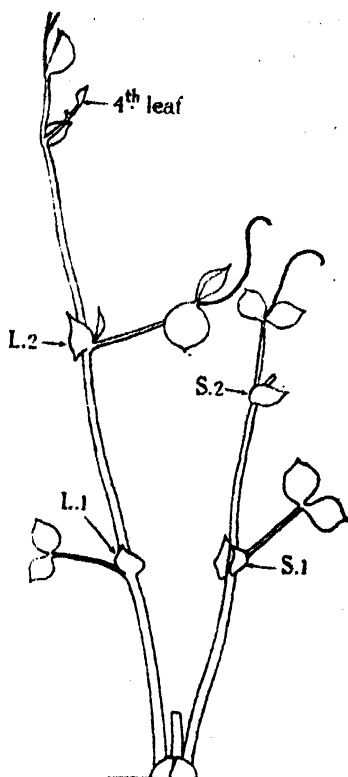


FIG. 2.—Illustrating experiment 5. Explanation in text.

The mean values of the growth of the buds, which were measured after times varying from 3 to 5 days, but naturally after the same time in each plant, were, for bud (1) 3.517 mm., and for bud (2) 3.403 mm. Thus the difference between the rates of outgrowth of these two buds is very slight, if any, when there are no developing leaves. Such slight difference as was found was in favour of bud (1), but it is not significant.

It is therefore clear that the fact that in experiment 5 L2 usually grew out before L1, must have been due to the effect of the young developing fourth leaves. For even if, on the basis of the controls, the chances of growing out first are taken to be equal for the two lowest buds of these shoots when no developing leaves are present, then simple calculation shows that the excess of the number of times in which L2 grew out first (that is, 28 times out of 36 instead of 18 times out of 36) is statistically fully significant.

The difference in mean rate of outgrowth between L1 and S1 was much greater still. It was indeed so great, that it can hardly be doubted that it is significant and due to the presence of the young fourth leaves on the longer shoots. For except as regards their distances from these leaves, the buds L1 and S1 were similarly situated, being corresponding buds on shoots of the same age, though admittedly the shorter shoots were less strongly developed than the longer ones.

On the other hand, the difference between the rates of outgrowth of S1 and S2, as shown by the greater number of priorities of the former bud, is not statistically significant, though it is suggestive.

The results thus show that the young developing leaves altered the relative rates of outgrowth of the buds in such a way as to give to L2, the bud nearest to them, an advantage over L1, the next bud, and to L1 an advantage over S1 and S2. Yet at the same time these leaves were retarding the growth of all the buds; for even L2, the most rapid of them, grew out very much less rapidly than the buds of the control plants of experiment 6 which had no developing leaves. It therefore appears that the young leaves inhibited least strongly the L2 buds, which were the nearest to them, more strongly the L1 buds, and most strongly of all the buds S1 and S2 on the other shoots, which were the farthest away. Thus once more it appears that the strength of inhibition increased with distance.

For the present, however, only the behaviour of the buds L2 and L1 will be considered to be evidence for the increase of inhibition with distance. For when L1 is compared with the buds on the shorter shoots, S1 and S2, it has to be remembered that in the shorter shoots inhibition was travelling upwards.

And it is possible that when inhibition crosses over in this manner from one shoot into another, its effectiveness may thereby be somehow increased. This point will be considered later in the light of some other experiments. The reason for performing this experiment on plants with two shoots was that it was hoped to discover, by comparing the growth of S1 and S2, whether inhibition increases with distance when acting upwards as well as when acting downwards. Actually the difference found between S1 and S2 was not statistically significant. But they were both so far distant from the inhibiting leaves, that the difference between their distances was relatively small.

The results already reported all tend to show that the strength of inhibition is made greater by a greater intervening length of stem, and some of them, those of experiments 1, 2 and 5, could not at all plausibly be explained in any other way. Account must also be taken of some results obtained by Child ((1), p. 292, and (2), p. 158), when performing the experiment of isolating axillary buds of *Phaseolus* from inhibition by placing round the stem above them a coil of tubing in which water at low temperatures, between 2° and 5° C., was circulating. He found that at the higher temperatures, e.g., 4° to 5° C., the cooled zone did not always release the buds below from inhibition, and that the farther away it was from them the less effectively did it do so. "These facts," he writes, "suggest that with the higher temperature the inhibiting factor is not completely blocked, and that in the normal parts of the stem below the cooled zone it gradually recovers its normal intensity or effectiveness." He also points out, however (*loc. cit.*), the possibility of another (not very probable) explanation, and in a recent letter to the writer he has been so good as to explain his views further. In this he states that he regards still a third explanation as possible, that is, that when the tubing is placed farther away from the buds and consequently higher up the stem, it surrounds a part of the stem which may be for some reason less susceptible to the effect of cold. It is thus certainly possible by means of such an assumption, to explain Child's results otherwise than as showing an increase of inhibition with length of stem. None the less, as he points out, they distinctly suggest that there is such an increase, and to this extent therefore they support the results here reported. Child considered, indeed, that the increase of inhibition with distance, if it was genuine, took place as a recovery or return to normal strength after inhibition had been somehow diminished, as for instance in passing a cooled zone. But, for reasons that will be made clear below, it is probable that the increase takes place quite generally in intact shoots also.

When the various experiments, all pointing in the same direction, are

considered together, there seems to be no room for doubt that the strength of inhibition really is increased by a greater intervening length of stem. It remains, however, quite possible that the increase may continue only until some certain distance is reached, and then cease. This conclusion, which may at first seem surprising, will be briefly discussed at the end of this paper.

But first it will be shown that as soon as the increase of inhibition with distance is recognised, it becomes possible to explain very simply a well-known fact that had previously remained quite unexplained—that is, the fact that as a rule axillary buds grow at first until they reach a certain length, about 1 mm. in pea seedlings, before they are stopped by inhibition. From this it might at first appear that in their youngest stages the buds are for some unknown reason not susceptible to inhibition. This, however, is not so, as the following experiment will show.

Experiment 7.—Six pea seedlings, about 45 mm. in height, were decapitated above the third leaves, which were already mature, and all visible buds were removed from the axils of the three remaining leaves. For this purpose, it was necessary to remove from the first and second axils the second smaller bud also, which is regularly formed beside the chief axillary bud. Six other seedlings, of the same batch, were similarly deprived of all visible buds in the axils of the three lowest leaves, but were left otherwise intact.

After 4½ days, it was found that in all the six decapitated seedlings, new buds had been formed in the axils of the first leaves and had grown to lengths from 2 to 3 mm. Also in four of these seedlings, new buds, rather smaller, had been formed in the second axils, and in four they were found (slightly smaller again) in the third axils. Meanwhile in the six seedlings with intact main shoots, no visible new buds had appeared in any of the three lowest axils, except in one seedling, in which one very small bud, of about 0·25 mm., was found in the second axil.

After 12 days, in the six latter seedlings, most of these axils were still without visible buds, though in some of them very small buds, of about 0·25 mm. had appeared. Meanwhile, in the decapitated seedlings, many of the new axillary buds had grown to leafy shoots up to 80 mm. in length. Another similar experiment gave similar results.

These experiments show that in seedlings with intact main shoots, if the buds are removed from the axils of mature leaves, new buds are formed in them only extremely slowly or not at all. The reason for this is clearly that they are inhibited by the apical region of the shoot, since in the corresponding axils of decapitated seedlings new buds are formed rapidly and grow out.

It is thus clear that axillary buds are susceptible to inhibition in their youngest stages, just as at other times.

If now one considers again the seedlings with intact main shoots, one sees that though the formation and early growth of new buds in their lower axils was strongly inhibited, yet, since the apical regions were growing normally, buds must have been appearing in the axils of the young leaves near the apex in the usual way, as was indeed observed. It therefore appears that in these shoots inhibition was again stronger at greater distances from the apex, and weak or absent at short distances. Such, at least, is the most simple explanation of the facts, which thus constitute another piece of evidence tending to show that the stem-length effect is genuine.

In the light of the last experiment, it is now sufficiently clear how the early growth of axillary buds in normal intact shoots is to be explained. They are normally formed in the axils of the youngest leaves of the apical bud, and are therefore for some time so close to the young rapidly developing leaves near the apex, which are the inhibiting members, that they cannot be inhibited by them. For some time, therefore, they are able to grow. But soon, through the continual growth of the apical bud and main shoot, they are passed back from the apical bud to distances farther and farther below it. As soon, therefore, as they become separated from the developing leaves near the apex by a sufficient length of stem, they are inhibited by them and grow no more.

It is natural to enquire at what distance from the apex inhibition begins to act effectively upon the axillary buds. The buds in the axils of the third leaves in experiment 1 had mostly reached very nearly, but not quite, the same size as those in the lower axils at the start, and they were at distances from 5 to 15 mm. below the young fifth leaves, which still formed part of the apical buds. Hence it may be estimated that at a distance of 15 mm. below the apical bud, the axillary buds have usually ceased to grow. But it does not follow that this is the distance at which inhibition first acts effectively upon them, for it is probable that some time elapses between the date at which inhibition begins to act effectively upon them and the date at which they cease to grow. Consequently it can only be said at present that inhibition begins to act effectively at some distance not greater than 15 mm. and probably less.

When it is recognised that the strength of inhibition increases with distance, some light is thrown also on the question raised by Child and Bellamy (3), p. 265) how it is that the apical bud, which inhibits other buds, does not inhibit itself also, or, to speak more strictly, does not inhibit itself completely, for it

is possible that some parts of the apical bud may retard other parts of it to some degree. This question is really complicated and involves several closely related questions which it is proposed to consider in another paper. Here, however, it may just be pointed out that since the apical bud inhibits axillary buds that are about 20 mm. distant less strongly than those that are four times further away or more, and since it inhibits those that are still closer less strongly again or not at all, it is perfectly in keeping with these facts if it inhibits only slightly or not at all that bud which is the closest of all to it, that is, itself.

Discussion.

The conclusion that the strength of inhibition increases with the length of intervening stem, for a considerable distance at least, is certainly at first surprising. In particular it may at first seem to be inconsistent with the conclusion drawn from earlier experiments ((4), experiment 8, and (5)) that in the transmission of inhibition the movement of a soluble substance formed by the inhibiting parts is somehow involved. Certainly it makes it difficult now to suppose that the transmission of inhibition consists *simply* in the movement of an inhibiting substance from apical bud to axillary. But the direct conclusion that followed from the earlier experiments was merely that *one stage* in the transmission of inhibition is the movement of a soluble substance away from the apex and towards the axillary bud. And this conclusion is not incompatible with the present results; for it remains possible that the whole process of downward transmission may be complex and comprise other stages also, as was clearly pointed out previously ((4), p. 857). And one of these other stages may be responsible for the increase of inhibition with distance.

Moreover, not only may the downwardly transmitted effect be itself complex but even if it is not, it may check the axillary buds in some way that is quite indirect. For instance, it may check them by modifying the conditions in the main shoot in some way that is unfavourable to their growth—perhaps by somehow causing some growth—promoting substances to flow up the shoot to the apical region and to accumulate there in higher concentration than lower down. A similar possibility was indeed considered previously ((4), pp. 856–857). It would follow that the conditions would be less and less favourable for the growth of axillaries, the farther distant they were from the apex. This suggestion is clearly quite different from the old and often criticised theory that the inhibition of axillaries is somehow directly due to the consumption of nutriment or other growth-promoting substances by the apical region, a theory

which would lead one to expect that the axillaries *nearest* to the apical region would be the most strongly inhibited.

When the various possibilities are considered, it can be seen that the increase of inhibition with distance is far from being incredible. But further discussion of it will be postponed until the results of certain other experiments have been reported.

Summary.

(1) In etiolated decapitated pea seedlings, single developing leaves inhibit axillary buds that are from 70 to 100 mm. below them more strongly than similar leaves in similar seedlings inhibit buds that are only from 5 to 15 mm. below them.

(2) Similarly apical buds, whose inhibiting effect has been weakened by removal of their outer leaves, inhibit axillary buds that are from 70 to 170 mm. below them more strongly than similar apical buds in similar seedlings inhibit those that are only from 14 to 42 mm. below them.

(3) In decapitated pea shoots, the effect of single developing leaves on two axillary buds at different distances below them is, that while they retard the outgrowth of both buds, they retard the more distant bud the more strongly.

(4) It is concluded that the strength of inhibition increases with increasing length of intervening stem.

(5) It is pointed out that axillary buds normally grow to a certain length before they are stopped by inhibition, and it is shown that, none the less, they are susceptible to inhibition during their early growth as well as later.

(6) It is concluded that the axillary buds are at first not inhibited because they are too close to the developing leaves in or near the apical bud. They therefore grow until, through the growth of the main shoot, they reach a distance below the developing leaves at which inhibition is strong enough to stop them.

(7) The interpretation of the increase of inhibition is briefly discussed.

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*The Combination Curves, Hydrogen Ion regulating Power and
Dissociation Constants of Gelatin.*

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The existing titrations of gelatin with acid and alkali have been found to refer mainly to solutions containing approximately 1 per cent. of the dry material, while the few titrations in 0.1 per cent. solutions are not in agreement. A series of titrations with alkali at both concentrations of gelatin has been made with a view to deciding whether the combining weight is really affected by the concentration. From the collected data, the combining or equivalent weight has been calculated by a new method based on the theory of buffer mixtures, and incidentally, the buffer capacities at the different concentrations have been determined. The equivalent so found has been compared with those which have been deduced by other methods. The equivalents of the 1.0 per cent. and 0.1 per cent. solutions in the alkaline range have also been calculated by another method using constants, and found to differ from one another. The equivalents as acid and as base have been deduced in various ways from the combination curves and also from the cleavage products, and these acid/base ratios have been compared. Finally by treating gelatin as a simple ampholyte, apparent constants have been calculated both on the undissociated molecule or older and on the "amphoteric ion" or newer theory.

Although gelatin is undoubtedly amphoteric in its nature, yet its curves of combination (p_H plotted against x = added acid or alkali) differ greatly from those of simple amino-acids such as glycine, as is easily seen by comparing the types which have been portrayed by Harris (1923, 1925, 1930).

Glycine has a minimum buffer capacity around its isoelectric point (pI) which is calculated to be 6.1 in the usual manner from $pK_A = 9.75$ (negative exponent of acid constant) and $pK_W - pK_B = 2.43$ (pK_B = negative exponent of basic constant). The curve runs sharply here through several powers of (H^+), on the addition of minimal amounts of acid and alkali. Gelatin, on the other hand, has a *maximum* buffer capacity over a range which includes the pI , 4.7. A knowledge of individual dissociation constants is not required

in the calculation of buffer capacity since this is simply determined from the slope of the curve, being, in fact, proportional to its cotangent, $\Delta x/\Delta p_H$, as plotted in the figure. Any buffered portion can be reproduced by *one* constant, which may be considered either as acidic or basic. From this point of view, gelatin can be treated as an acid having pK_A in the neighbourhood of the pI , e.g., at $p_H = pK = 4.5$. Moderate variations in the volume of the solution do not appreciably affect the acid and alkali requirement (hydrion or hydroxyion error on Bjerrum's theory) provided that the p_H lies between 3.5 and 10.0. Therefore the buffer capacity of 1 g. of gelatin can be defined without specifying volumes, as is shown by the substantial agreement of the middle parts of the curves referring to the two concentrations.

Buffer Capacity and Equivalent Weight.

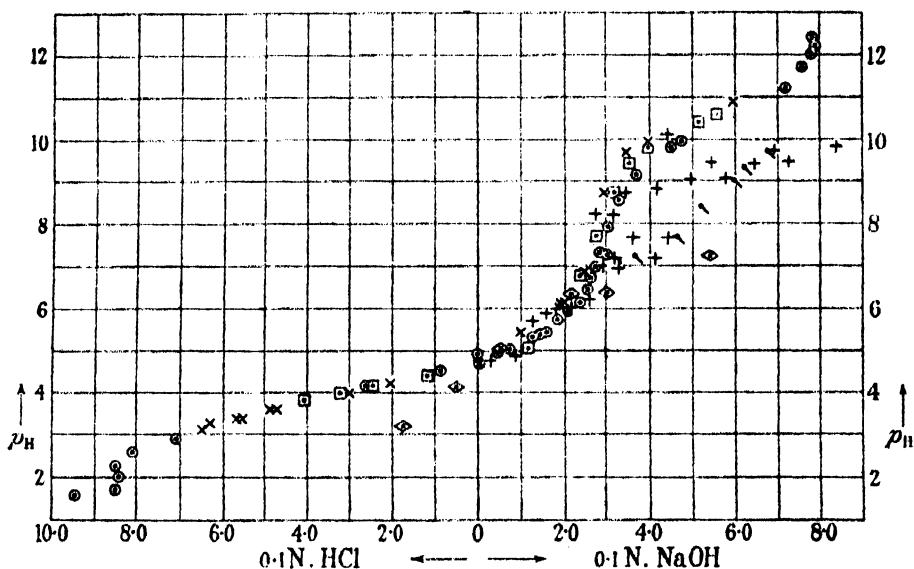
If Δv denotes the number of molecules of 0.1 N acid or alkali added to 1 g. of gelatin in solution at about $p_H = 4.5$, then, by measurement from the graph, $\Delta v/\Delta p_H = 3.9$. If Δc = equivalents of acid or alkali, then $\Delta v = 1 \times 10^{-4} \Delta c$, and $\Delta c/\Delta p_H = 3.9 \times 10^{-4}$.

In the general theory of buffer mixtures (Clark, "Determination of Hydrogen Ions," chap. II) it is proved that $dx/dp_H = 0.576$ at the point of half neutralisation, when α degree of neutralisation = $1 - \alpha$. If c denotes the equivalent concentration of acid or alkali and of the gelatin, then $dx = dc/c$ and $dc/dp_H = 0.576c$. Thus for a 0.1 N electrolyte, $dc/dp_H = 0.0576$. This value is naturally much greater than that found in the case of 1 g. of gelatin. Its normality, considered as a monobasic acid is $3.9 \times 10^{-4}/0.576 = 6.78 \times 10^{-4}$. The equivalent weight is therefore 1470. Gelatin could be replaced, so far as buffer action is concerned, by an acid having $pK = 4.5$, and this equivalent weight.

The results of the titration of gelatin by various investigators have been reduced to a uniform standard laid down by Loeb and others, i.e., number of ml. of 0.1 N acid or alkali added to 1 g. of originally isoelectric gelatin. These volumes where necessary have been corrected for 'acid' or 'alkali' blank according to the tables published by Harris (1923) or Atkin and Douglas (1924). The figure contains the results of Loeb, "Proteins and the Theory of Colloidal Behaviour," chap. IV, Table I, corrected further for acid blank, of Atkin and Douglas (*loc. cit.*), of Simms (1923), and of Jermolenko (1929).

The titrations of Loeb and Atkin and Douglas were carried out with hydrogen electrode, the latter referring to a temperature 24° or 25°. The other half cell was a saturated KCl calomel electrode. Simms also used a hydrogen

electrode. His results are expressed in an unusual manner and when multiplied by 4 appear to be converted to the present standard. The investigation of



The combination of 1.0 and 0.1 per cent. gelatin with acid and alkali.

× × Loeb, 1 per cent. in acid solution from table, corrected by acid blank and from smoothed values. Loeb 0.8 per cent. in alkaline solution from graph. ● ● Atkin and Douglas, 1 per cent. □ □ Simms, 1.25 per cent. ◊ ◊ Jermolenko, 0.1 per cent. with indicators. + + Author, 0.1 per cent. with hydrogen electrode. \ \ Mr. F. O. Howitt.

Jermolenko (1929, *loc. cit.*) was mainly concerned with surface tension but also furnishes titration values of a 0.1 per cent. gelatin solution, in which the hydrogen ion concentration was determined by indicators. When corrected to ml. acid or alkali from the *pI* and reduced to the same standard, these results are seen to deviate widely in some cases from those in the 1 per cent. solutions. In particular, the diminution in alkalinity of the sodium gelatin solutions appears to be too great to be due to reversible hydrolysis alone.

This part of the curve was, therefore, determined, and in the following manner.

Experimental.—The gelatin, prepared according to the method of Loeb, was similar to that described in a previous communication, Prideaux and Howitt (1929). 5.3 g. of a stock solution which contained 0.1 g. of dry gelatin was liquefied and made up to 100 molecules with distilled water free from CO_2 . Titrations with 0.0795 N alkali were carried out by means of a dipping hydrogen

electrode; the other half-cell being a saturated KCl calomel electrode. The cells were immersed in a thermostat at 25° and connected by salt-bridges of saturated KCl and agar. This combination was placed in series with, and in opposition to, a cadmium cell, and the e.m.f.'s were read on a Cambridge potentiometer. The cadmium cell was checked at frequent intervals against an N.P.L. standard. If the working standard showed 1.0182 volts on one day, then the normal potential ($H = 1$) of the whole combination was 1.0182 — 0.2464 (equals the single potential of the saturated calomel half-cell at 25.0° C.). If E is the e.m.f. corresponding to the unknown p_H , then

$$p_H = \frac{0.772 - E}{0.059}$$

The hydrogen was passed until a steady potential was obtained, which required at least an hour in the early stages, but a shorter time in the later stages of a titration.

50 ml. of the 0.1 per cent. solution were taken. A slight excess, say 0.05 ml., of the 0.1 N acid was added from a micropipette, and then the 0.1 N alkali from a microburette, up to about 0.4 to 0.5 ml., to give p_H between 9 and 10. On graphing the results, the volume of acid required to change the natural p_H to the $pI = 4.7$ was found. This was about 0.5 molecule per 1 g. of gelatin, in agreement with the results of Douglas and Atkin (*loc. cit.*). The excess of acid over and above this amount was, of course,

Alkali Titration of 0.1 per cent. Gelatin.

ML. 0.1 N. NaOH combined with 1 g. of isoelectric gelatin.	p_H .	ML. 0.1 combined isoelec	of p_H .
I 0.217,	4.78	4.56	7.66
1.78	6.05	5.75	9.50
2.90	6.99	7.40	9.70
II 2.80	8.24	5.97	9.10
3.58	8.70	6.77	9.39
4.23	8.79	7.54	9.54
5.16	9.04	9.11	9.83
III 1.30	5.72	3.36	6.90
1.61	5.89	4.16	7.18
2.09	6.03	4.95	8.55
2.57	6.20	5.59	8.85
IV 3.70	7.23	6.10	9.00
4.60	7.60	6.20	9.30
5.30	8.40	6.80	9.70

subtracted from the alkali titrations. The series quoted were each determined on separate days with freshly made solutions. The fourth series was determined by Mr. F. O. Howitt, using an entirely different outfit and with a very long continued passage of hydrogen.

From the pI up to $p_H = 7.0$ the curve follows closely that of 1 per cent. gelatin. At this point (or 8.7 in Series II) the curve begins to diverge in the direction which is to be expected on account of hydrolysis. In the range p_H 7 to 9, series II, does not agree with the others; values of $[H^+]$ do not seem well defined in this part. Small differences in the degrees of hydrolysis will here greatly affect values of $[H^+]$ or $[OH^-]$ which are still quite low. From 9.2 upwards the different series agree better, and a steady divergence of nearly 2.0 in p_H is found between the 1.0 and 0.1 per cent. solutions containing the same ratio of alkali to gelatin.

The 0.1 per cent. solution has a far greater buffering power per 1.0 g. of gelatin than the 1 per cent. solution. Thus, about 3.5 ml. of 0.1 N alkali are required to change p_H from 4.7 to 9.0 in the 1.0 per cent. and about 6.0 ml. of the alkali to produce the same change in the 0.1 per cent. solution. This difference is not due to alkali blank error, which has been allowed for, and is quite low at $p_H = 9.0$.

Equivalent Weight of 0.1 per cent. Gelatin in the Alkaline Range.

If it were possible to locate a second inflection in the 0.1 per cent. solution corresponding to that which occurs at about $p_H = 12$ in the 1.0 per cent. solution, then the equivalent could be calculated directly from the amount of alkali required. This is not possible, however, on account of high hydrolyses. The calculation will be made by another method using equations published in 1915.

The constants will be considered as acidic constants, for purposes of calculation. By using the basic constant derived from the titration with acid, and applying the Walker-Michaelis equation

$$p_H = \frac{pK_W - pK_B + pK_A}{2} \text{ (at } pI\text{).}$$

Atkin and Douglas (*loc. cit.*) calculate that $pK_A = 5.7$ or the first acidic constant K_1 is 2×10^{-6} .* This supplies a basis for the calculation of the second, or weaker, acid constant K_2 , or the basic constant K_B . For at the middle of the first inflection which is found to vary only slightly with concentration,

* This is called the second constant on p. 232 for obvious reasons.

$p_H = 7.7$. This is the first equivalence point, at which all the stronger acidic groups are neutralised. The usual expression for K_1 and K_2 gives at this point

$$p_H = 7.7 = \frac{pK_1 + pK_2}{2} = \frac{5.35}{2} + \frac{pK_2}{2}.$$

Hence

$$pK_2 = 10.05, \quad K_2 = 1 \times 10^{-10}.$$

The general equations of a dibasic acid may now be applied at any convenient point in the neutralisation curve beyond the first equivalence point. After the addition of 6 ml. of 0.1 N. NaOH to 1 g. of gelatine, the p_H values in the 1.0 and 0.1 per cent. solutions are 10.8 and 9.0 respectively. The equation

$$R = \frac{2 + [H^+]/K_2 + K_w (K_2 + [H^+])/[H^+] K_2 C}{1 + [H^+]/K_2 + \frac{[H^+]^2}{K_1 K_2}}$$

is solved for the two cases. R is the ratio, equivalents of alkali (equals ml. of 0.1 N alkali $\times 10^{-4}$) to ml. of gelatin (equals weight $\div$ unknown molar weight M). Since the molar weight may vary with the dilution, M_1 and M_2 may be written for the values in 1.0 and 0.1 per cent. solution. Hence, in the 1.0 per cent. solution

$$R_1 = 6 \times 10^{-4}, \quad p_H = 10.8, \quad [H^+]_1 = 1.6 \times 10^{-11}, \quad c_1 = 10/M_1,$$

and in the 0.1 per cent. solution

$$R_2 = 6 \times 10^{-4}, \quad p_H = 9.0, \quad [H^+] = 1 \times 10^{-9}, \quad c_2 = 1.0/M_2.$$

On putting in the numerical values, with $K_w = 0.64 \times 10^{-14}$,

$$M_1 = 3280 \quad \text{and} \quad M_2 = 1980.$$

The first agrees fairly well with the molar weight 3290 of 1 per cent. gelatin obtained by Atkin and Douglas, who used a different method (see note on p. 230).

Since a lower equivalent weight is found in the more dilute solutions, by a calculation which takes full account of hydrolysis, the lower p_H values in the 0.1 per cent. combination curve are not due to increased hydrolysis alone. Further calculations, using the same equations at other points in the curves gave similar results. Thus, at $p_H = 8.3$, R may be between 5.0 and 3.9. Hence M_2 is 2030 or 2060.

The fluctuating values in the titration curve between 3 and 6 molecules of

alkali and at $p_H = 7.0$ to 9.0 are possibly due to an irreversible hydrolysis, as was shown by an experiment designed to test this possibility.

Relation between the Equivalent Weights as Acid and as Base.

According to the newer theory which has been explained in detail by Harris and co-workers (*loc. cit.*), the combination of ampholytes with acids corresponds to the displacement of carboxyl groups, while the combination with alkalis corresponds to the displacement of basic groups. The constants will be expressed both on the newer and on the older theory.

From the Constitution.—An estimate of the separate cleavage products, including a small proportion of ammonia, which have actually been obtained from gelatin, and which account for 91.3 per cent. of the material, is given in "Practical Organic and Biochemistry," Plimmer, chap. 39.

Taking into account the dicarboxy and the dibasic amino acids, a total is obtained of 91.9 molecules of acidic radicals and 98.4 of basic radicals. The ratio, equivalent weight as base to that as acid, is therefore 0.93.

(2) *From the Equivalents Directly obtained by Titration.*

These have been stated by Atkin or deduced from his curves. Starting from $p_H = 4.7$ the limit beyond which no acid is fixed, at $p_H = 1.7$, is 8.5 ml. of 0.1 N reagent per 1 g. of gelatin, therefore the equivalent as base on the older theory is 1180. The titration to the limit beyond which no alkali is fixed, is 7.8 molecules of 0.1 N reagent, therefore the equivalent as acid is 1280.* Ratio 0.92. On the newer theory the equivalents are reversed and the ratio is 1.08.

(3) *From the Equivalents Obtained by the Use of the Buffer Equation and Constants.*

The equivalent 1470 calculated from buffer action at or below $p_H = 4.7$ corresponds most nearly on the older theory to the basic function. The molar weight of gelatin considered as containing two acidic groups is 3280 (see above) or the equivalent is 1640. Thus the ratio on the older theory is 0.90, while that on the newer theory is 1.10.

Thus the older theory agrees better on the whole with the constitution of gelatin than the newer. It is significant that by either method of calculation, the equivalent weights as acid and as base are nearly equal. This points to

* Atkin and Douglas (*loc. cit.*) obtain 3290 as the first equivalent up to $p_H = 7.7$, but the second acid equivalent requires more alkali than the first so that the mean equivalent weight is less than 3290/2.

the opinion that the original molecule has nearly equal proportions of those cleavage products—arginine and glutamic acid—in which the number of acidic is not equal to that of basic groups.

The Probable Value of the Constants on the Newer Theory of Ampholytes.

The absence of inflection at the isoelectric point is, as already shown, connected with the relative magnitudes of the two constants involved. For the sake of simplicity these will first be considered as the constants of a dibasic acid which are numbered from the acid side so that K_1 refers to the back titration of the strongly acid hydrochloride with alkalis.

It has been proved by Auerbach and Smolczyk (1924) that there is no inflection in the combination curve at the equivalence point of the first acid, when $K_1 \leq 16K_2$, or $pK_1 - pK_2 \leq -1.204$. Thus, in the case of tartaric acid, $pK_1 = 2.90$ and $pK_2 = 4.02$ and there is no inflection, while in that of oxalic acid, $pK_1 = 0.77$ and $pK_2 = 3.86$, there is one.

We may put this limiting ratio at 20 or even higher in the case of a protein and still not have a perceptible inflection. The constants deduced by Douglas and Atkin (*loc. cit.*) and still considered as acid constants are— $pK_1 = 3.7$ and $pK_2 = 5.7$. The ratio K_1/K_2 is 100 and the difference between the exponents is 2.0 instead of 1.3 ($-\log 20$), so that there should be a perceptible inflection. These constants may be revised, and both determined by the application of the two equations

$$pK_2 - pK_1 \approx 1.3 \quad pK_2 + pK_1 = 2 \times 4.7.$$

Thus

$$pK_2 \approx 5.35 \quad \text{and} \quad pK_1 \approx 4.05.$$

We may omit the sign of inequality, since it is unlikely, on the analogy of the other amino-acids, that the ratio of K_A to K_W/K_B is less than 20.

If the pI is about 5.0, as seems to be indicated by some measurements of electrophoresis, then

$$pK_2 = 5.6, \quad pK_1 = 4.3.$$

Thus it appears that pK_2 can hardly be greater than 5.6, and may be less, and this requires that pK_1 should be 4.3 or 4.0. Either of these values agrees well with the acid combination curve, since the point at which 99 per cent. of the possible acid is combined may be at p_H 2.0 or 2.3* instead of 1.7 as assumed by Atkin and Douglas (1924), since these points are uncertain on account of the high "acid blank" correction.

The above results will now be translated into the newer or amphoteric ion

$$* p_H = [4.3 \text{ or } 4.0] - 2.0.$$

theory. Since the protein dissociates in such a way that the constant K_1 is that the K_A due to the displacement of the carboxyl groups by HCl, while K_2 is replaced by K_W/K_B in which K_B refers to the displacement of the weaker base by NaOH, then $pK_A - pK_W - pK_B = -1.3$.

The condition for absence of inflection may also be written

$$pK_A + pK_B = pK_W - 1.3 = 14.2 - 1.3 = 12.9 \text{ (at } 18^\circ\text{)}.$$

Taking the pI as 4.7 we have as before

$$pK_A = 4.3, \quad pK_B = 14.2 - 5.35 = 8.85.$$

It is not quite certain whether the third constant, which determines the dissociation in the more alkaline solutions, refers to an acid or a base. The latter view seems to be favoured by the formaldehyde titrations of Birch and Harris (1930). According to p. 228, the exponent of this constant is

$$pK_{B_1} = 14.2 - 10.05 = 4.05.$$

These electrochemical properties of gelatin may now be briefly summarised.

- (1) Isoelectric gelatin is assumed present as undissociated molecules

$$pI = 4.7, \quad pK_B = 10.15, \quad pK_{A_1} = 5.35, \quad pK_{A_2} = 10.15.$$

$$pI = 5.0, \quad pK_B = 9.9, \quad pK_{A_1} = 5.6, \quad pK_{A_2} = 9.8.$$

- (2) Isoelectric gelatin is assumed present as amphoteric ion.

$$pI = 4.7, \quad pK_A = 4.05, \quad pK_{B_1} = 8.85, \quad pK_{B_2} = 4.05.$$

$$pI = 5.0, \quad pK_A = 4.3, \quad pK_{B_1} = 8.6, \quad pK_{B_2} = 4.4.$$

It will be noticed that the dissociation constants of the stronger acidic are nearly equal to those of the stronger basic groups on the newer theory (2), while constants of the weaker acidic are nearly equal to those of the weaker basic groups on the older theory.

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A Comparative Study of the Brains in British Cyprinoids in Relation to their Habits of Feeding, with special reference to the Anatomy of the Medulla Oblongata.

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Introductory.

Professor G. H. Parker, writing in 1922, remarks that the comparative physiology of taste in Vertebrates is almost an untouched field.

It occurred to the writer of this paper, that it would be an instructive research to take a family, such as the carp family, and to study the hind brain of the members of the Cyprinidæ found in British waters, and to correlate their habits of feeding with the developments of the vagal and facial lobes of the *medulla oblongata*.

(As is well known, the Cyprinidæ and the Loaches (Cobitidæ) belong to the order Ostariophysii. They possess the interesting apparatus connecting the swim-bladder with the ear, known as the Weberian Mechanism.

The Cyprinidæ are also, I believe, peculiar in possessing a high pressure swim-bladder, in which, as in the Roach for example, the contained gases are maintained at an average pressure of 60 mm. Hg.

Another peculiarity of the Cyprinoids is the possession of the palatal organ in the roof of the pharynx, originally described by Weber in 1827. This remarkable organ is crowded over its entire extent with taste buds, arranged on comb-like ridges. (There are certain peculiarities in the configuration of the hind brain in Cyprinoids. "The vagal lobes in the Carp, *C. carpio*, form a pair of wing-like swellings on either side of the rhomboid fossa, and embrace between their anterior ends the unpaired facial lobe, the *lobus impar* or *lobus facialis*") ('Cat. Roy. Coll. Surgeons,' Eng.).

(It is known that, among Cyprinoids, the Goldfish has taste buds over the whole body, that the Barbel has taste buds on the barblets, and that they are also present on the surface of the Tench.)

(Herriek, writing in 1905 on the gustatory paths in fishes, states that "all taste buds in the pharynx and back part of the mouth are supplied by the vagal and glossopharyngeal nerves—those in front of the mouth, lips and outer

skin from a root of the facialis, which apparently corresponds with the *portio intermedia* of human anatomy.

Associated with these roots are unspecialised sensory fibres ending in the mucous membrane of the mouth. The central connections are not known—they are termed, provisionally, the *communis* system.

The specialised *communis* fibres related to taste buds, either within or outside the mouth, and the ganglion cells from which they are derived are termed "the peripheral gustatory system of neurones."

There is a special centre in the *medulla oblongata* for the reception of the facial root of the *communis*, termed the facial lobe. The size of this lobe appears to depend on the possession of a large number of terminal buds on the outer skin.

The enormous vagal lobes of some of the Cyprinoids are explained by the fact that these are the terminal centres for the nerve fibres of the X and XI nerves from the palatal organ. Siluroids have no hypertrophy of the gustatory organ in the branchial region, and, therefore, we find the vagal lobes as in most teleosts; but as the cutaneous sensory organs are very highly developed we find large facial lobes.

To quote Herrick again: "The vagal lobes (for mouth tasting) and facial lobes (for skin tasting) are local enlargements of the visceral sensory brain, as is illustrated in the dog fish." The behaviour of these fishes (Siluroids and Cyprinoids) has been studied, and interesting correlations between these remarkable sense organs and the methods of feeding brought to light.) It should also be mentioned that in a recent paper, Muir Evans and Damant (1928) have drawn attention to the presence, in the oesophagus of the Cyprinoids, of taste buds surrounding the entrance of the pneumatic duct; their presence there is doubtless associated with the swallowing of air by these fish, for the purpose of replenishing the gases in the swim-bladder.

Historical.

The naked appearance of the brains of these fish have been previously reviewed by P. Savourè (1912). The illustrations published by Savourè are on a small scale, and diagrammatic, and our observations do not agree with the descriptions of this writer in many instances. For example, the two Breams are figured, but the drawings of the vagal lobe are not in accordance with the conditions we find in *Abramis brama* and *Abramis bjoerkna* of the Norfolk rivers; neither do our observations agree with his description of *Gobio fluviatilis*.

(This author describes the large bilateral lobes of the *medulla*, as a posterior swelling called the "lobe posterieure" by Klaatsch and the pneumogastric lobe by Baudelot; between these lobes is a central lobe, the median lobe of Baudelot or "lobule mediane du Bulbe."

The terms now usually applied to these lobes are "vagal lobe" to the former, and "facial lobe" to the latter.)C. Judson Herrick in numerous papers and books, 1899, 1902, 1905, has made valuable and exhaustive studies of the gustatory paths in fish. He has made detailed examinations of the skin of *Amiurus melas*, a small cat fish, and has prepared a diagram, which has been reproduced in most text-books dealing with this subject, of the distribution of the taste buds over the external surface, and of the cutaneous branches of the VII cranial nerve which supply them (1903). (He states that in the Siluroids (Catfishes) the highly developed cutaneous gustatory organs, innervated by the facialis, have called forth an enlargement of the facial lobes greater than in that of the Cyprinoids.)

This generalisation does not, however, hold good; we shall see further, in the course of this communication, that in both the Barbel and the Gudgeon the facial lobe is as fully developed as in the Siluroids.

G. H. Parker (1922) has reviewed the question of taste in fishes in his book of "Smell, Taste and Allied Senses in Vertebrates."

Olmstead (1918) has made experiments on the nature of the sense of smell in the Common Catfish *Amiurus*. (He finds that taste-buds on the oral barblets begin to degenerate in a little over 10 days after the nerve to these organs has been cut)

Examination of the Brain in situ.

Group I.—Carp, Goldfish, Bream and Tench.—The vagal lobes are large, and oval or crescentic in shape. In the Carp, the facial or central lobe is not overlapped by the vagal lobes, as it is in the Goldfish. In the Bream, the facial lobe is small and is more anterior in situation than in the others of this group.

In the Tench, the facial lobe is large and causes a wide separation of the anterior ends of the vagal lobes. The acoustic tubercle is very similar in size in all four, and the cerebellum is very similar in size and shape in the Carp, the Goldfish, and the Bream; in the Tench it is broader than in the other members of the group.

Group II.—Roach, Rudd, Dace and Chub.—The optic lobes are large compared with the vagal and facial lobes. The vagal lobes are comparatively small and the facial lobes are small, especially so in the Dace and the Chub,

GROUP I.
Vagal Lobes Large.



FIG. 1.—Carp.
Cyprinus carpio.



FIG. 2.—Goldfish.
Carassius auratus.



FIG. 3.—Bream.
Abramis brama.



FIG. 4.—Tench.
Tinca tinca.



FIG. 5.—Roach.
Rutilus rutilus.



FIG. 6.—Rudd.
Scardinius erythrophthalmus.



FIG. 7.—Chub.
Leuciscus cephalus.



FIG. 8.—Dace.
Leuciscus leuciscus.

GROUP III.
Facial Lobes Large.



FIG. 9.—Barbel.
Barbus barbus.

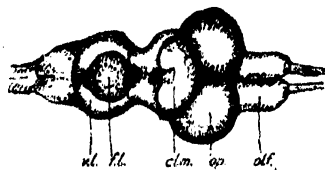


FIG. 10.—Gudgeon.
Gobio gobio.
N.B.—Cerebellum is raised up.



FIG. 11.—Loach.
Nemachilus barbatulus.

NOTE.—Details of the olfactory lobes and bulb are purposely omitted except in Carp and Dace. *at.*, acoustic tubercle; *clm.*, cerebellum; *fl.*, facial lobe; *olf.*, primitive end brain; *olf. b.*, olfactory bulb; *op.*, optic lobe; *pall.*, pallium; *s.c.*, spinal cord; *v.l.*, vagal lobe.

and, as in the Bream, situated at the anterior extremity of the vagal lobes. The cerebellum and acoustic tubercles are of moderate size.

Group III.—Consists of the Barbel and the Gudgeon, and in it is placed the Loach, because it is a near relative to the Cyprinoids, and its habitat and habits are very similar to those of the Barbel and the Gudgeon. In these three the vagal lobes are well marked. The facial lobe is much enlarged, especially in

the Gudgeon and the Loach ; it overlaps the vagal lobes in Gudgeon, and in the Barbel the large facial lobe separates the anterior ends of the vagals to a greater extent than in the Tench. The cerebellum is large and overlaps the central lobe. In the Barbel, the *valvula cerebelli* widely separates the optic lobes, and the same condition is noted in the Loach.

Barbels.

The presence or absence of barbels in these groups will now be considered.

In Group II, Roach, Rudd, Chub and Dace, there are no barbels.

In Group III, barbels are present in each fish.

The Barbel has two barbels on each side.

The Gudgeon has one barbel on each side.

The Stone Loach has a blunt snout, projecting beyond the transverse, inferior mouth, in front of which are two pairs of barbels, whilst a third pair are placed near its corners (Tate Regan) ; that is to say, it has three barbels on each side.

Group I.—The Carp, *Cyprinus carpio*, has two barbels on each side of the mouth.

The Crucian Carp and the Goldfish have no barbels.

The Bream has no barbels.

The Tench has a short barbel on each side.

Feeding Habits of Cyprinoids.

Although the feeding habits of British Cyprinoids are similar, in that they subsist on a mixed diet, some are almost entirely vegetarian, while others are predacious. They can, however, be divided into three groups according to their habits of feeding.

Group I.—Those fish presenting the type of hind brain figured in Group I, are the Carp, the Goldfish, the Bream and the Tench. All these fish can extract nourishment from mud. Yarrell gives the larvæ of insects, worms, and the softer parts of aquatic plants as the food of the Carp. Shrimps are also eaten. Bream swim in shoals, feeding on worms and other soft-bodied animals, with some vegetable substances (Yarrell).

Walton gives as baits for Bream, "paste of brown bread and honey, gentles or the brood of wasps that be young." "There is at the root of docks or flags or rushes in watery places, a worm not unlike a maggot, at which Tench will bite freely." But for Carp or Bream he recommends "as big a red worm as

you can find without a knot"; this must be carefully cleaned with moss that must be changed fresh every 3 or 4 days. I cannot forbear quoting Walton on the Tench: "This fish that carries a natural balance in him to cure both himself and others, loves yet to feed in very foul water amongst weeds"; "He will bite at a paste of brown bread and honey, or at a marsh worm or a lob-worm"; and, strange as it may seem, "he inclines very much to any paste with which tar is mixt." But the dominant characteristic of this group is the power of sifting mud, so as to extract nutriment from the organic matter which it contains.

Group II.—All the members of this group feed largely by sight. It includes the Roach, Rudd, Chub and Dace. All will take a fly, and the Rudd and the Dace give good sport to the dry-fly fisherman. The Dace feeds on insects, shrimps, worms and flies. The Roach feeds on weeds, insects, larvæ, little shell-fish and flies. The Rudd feeds like the Roach, and prefers still, weedy places. The Chub leaps at flies, or feeds at the bottom on weeds, shrimps, worms, or young frogs, and also preys on Minnows and Gudgeon. According to Isaac Walton the Chub will take a grasshopper. He recommends as baits "a black snail with his belly slit to show his white, or a piece of soft cheese. Nay, sometimes a worm or any kind of fly as the ant-fly, the flesh fly or wall fly or the dor or beetle you may find under cow dung, or a lob which you will find in the same place and in time will be a beetle; it is a short, white worm like to and bigger than a gentle."

Group III. Feeding Habits of the Gudgeon and the Barbel.—These are well described by Isaac Walton. The Gudgeon frequents gravelly bottoms. "The Germans call him Groundling, by reason of his feeding on the ground and on the gravel; and he there feasts himself in sharp streams and on the gravel." "He and the Barbel both feed so and do not hunt for flies at any time as most other fishes do." "He is easily taken with a small red worm." "The food of the Barbel is partly of an animal and partly of a vegetable nature. He does not disdain any sort of vegetable matter which he finds by rooting about on the bottom or banks with his snout, often turning over stones and using the barbels as tasters in search of food."

In general, it may be said that their diet is one of shrimps, small molluscs, insect larvæ, worms and eggs or fry of other fish.

"The Barbel love to lurk under weeds and to feed on gravel against a rising ground, and will root and dig in the sands with a nose like a hog. He is also curious for his baits; that they be clean and sweet; at a well scoured lob-worm he will bite boldly and also at gentles not too much scoured; cheese is

also a good bait, if it were laid in clarified honey a short time before as namely, an hour or two."

The habits of these two fish are therefore remarkable for their similarity, in that they grope and grub for their food and never take a fly, and, therefore, it is not surprising that their hind brain (both facial and vagal lobes) is peculiarly developed.

Technique of Histological Examinations. X

The fish was killed with chloroform and the brain removed and fixed in Bouin's fluid 12 hours. Bouin's fluid is composed of the following :—

Saturated aqueous solution of picric acid	15 parts
Formalin	5 parts
Glacial acetic acid	1 part

It was passed into 50 per cent. alcohol for 24 hours, and then into 70 per cent. alcohol, changing every 3 hours for 2 days, to remove all Bouin's fluid. Then 90 per cent. alcohol for 2 hours, followed by two changes of absolute alcohol for 1 hour each. The tissue was cleared in cedar-wood oil and embedded in paraffin.

The sections were passed through two changes of xylol and then through descending strengths of alcohol into distilled water. They were then stained with Delafield's hæmatoxylin for 5 to 10 minutes and then passed through 30 per cent. and 50 per cent. into 70 per cent. spirit. They were counter-stained with picro-indigo-carmin for 5 seconds, and finally passed through 90 per cent. spirit and absolute alcohol, into xylol, and mounted in Canada balsam.

The formula of picro-indigo-carmin used :—

- A. Saturated solution of picric acid in 90 per cent. alcohol.
- B. Saturated solution indigo-carmin in 70 per cent. alcohol in the proportion : A, 1 vol., B, 1 vol., 70 per cent. alcohol, 6 vols.

The Histology and Comparative Microscopic Anatomy of the Medulla Oblongata of Cyprinoids.

In order to make clear the difference of microscopical structure in the brains of the three groups of Cyprinoids, which, as we have already seen, are distinguished by naked eye characters correlated with their habits of feeding, it will be necessary to recall some facts in the histology of the *medulla oblongata*. Especially, we must recall the recent work of C. J. Herrick on the vagal and facial lobes, which form part of his elaborate study of the gustatory tracts in

fishes. We, therefore, in the first place, reproduce his diagrammatic section of the *medulla* of the bony fish the Sea-robin, *Prionotus carolinus*, in the region of the vagus nerve.

The four principal functional systems are arranged in longitudinal columns from the dorsal to the ventral surface in the following order : Somatic Sensory, Visceral Sensory, Visceral Motor, and Somatic Motor columns as indicated diagrammatically on the left side of fig. 12.

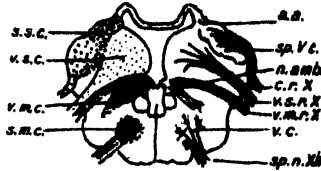


FIG. 12.—Diagrammatic cross-section through the *medulla oblongata* at the level of the vagus nerve in a bony fish (the Sea-robin, *Prionotus carolinus*, to illustrate the arrangement of the four principal functional columns from Herrick, "Introduction to Neurology." *a.a.*, acoustic area; *c.r.X.*, cutaneous root X; *n. amb.*, nucleus ambiguus; *s.m.c.*, somatic motor column; *sp. n. XII*, spinal nerve XII; *sp. V t.*, spinal V tract; *s.s.c.*, somatic sensory column; *v.c.*, ventral column; *v.m.c.*, visceral motor column; *v.m.r.X.*, visceral motor root X; *v.s.c.*, visceral sensory column; *v.s.r.X.*, visceral sensory root X.

The arrangement of the peripheral nerve-fibres is indicated on the right side of the diagram.

The Medulla Oblongata of the Carp.—The catalogue of the Museum of the Royal College of Surgeons thus describes the *medulla oblongata* : "The vagal lobes form a pair of wing-like swellings on either side of the rhomboid fossa in the Carp, and embrace between their anterior ends the unpaired facial lobe; this *lobus impar* or *lobus facialis* represents apparently a fusion of the two small facial lobes in the cod." That this suggestion is correct is proved by sections of the medulla in the Gudgeon, where the two halves of the facial lobe appear distinctly separated at the level of the entrance of the facial nerves.

Fig. 13 is a section across the *medulla oblongata* of the Carp at the level of the entrance of the X nerve, the three roots of which are clearly evident on the left side of the section. The enormous development of the sensory and motor columns is remarkable, but their relative relations in position remain the same as in *Prionotus carolinus*, when allowance is made for the distortion produced by their size.

On the left of the section are to be seen the cutaneous root of the Xth passing

to the somatic sensory columns, which embraces the whole lobe ; the visceral sensory root of the Xth is seen passing to the visceral sensory column, and the visceral motor root is seen passing medially to the *nucleus ambiguus*.

On the right, however, the root of the somatic sensory column does not appear in the section. The great longitudinal secondary gustatory tracts are seen on either side.

The *substantia reticularis* appears between the *fasciculus longitudinalis medialis*, and the secondary gustatory tracts, and ventral and external to the *fasciculus medialis* is the ventral funicle of the spinal cord. The motor cells of the *nucleus ambiguus* are very well marked.

On the left, on the periphery of the somatic sensory column is a layer of chief secondary gustatory neurones ; on the right side on the periphery of the sensory somatic column is to be noted the superficial root of the Xth, internal to which is a layer of secondary gustatory neurones.

Three divisions can be recognised in the great longitudinal tracts on either side, the spinal root of the V, the descending secondary gustatory tract of the facial, and the ascending secondary gustatory tract, as one passes from the dorsal to the ventral aspect. These divisions are more clearly shown in sections from the Tench.

The explanation of this enormous development of the vagal lobes in the Carp and other mud-eating members of the Cyprinidæ is " the existence of the palatal organ in the roof of the pharynx, which is very thickly covered with taste-buds arranged on comb-like ridges " (Herrick).

During feeding, this is enlarged by congestion with blood, and serves to sort out food particles from the mud.

" The vagal lobes for mouth-tasting are therefore local enlargements of the visceral sensory brain, as it exists in the Dog-fish, and in bony fishes, as exemplified by the Sea-robin " (Herrick).

The motor layer of the vagal lobe is regarded as a dorsal extension of the *nucleus ambiguus*, the motor fibres from which pass out chiefly to the small muscles which permeate the interior of the palatal organ.

The *nucleus ambiguus* has the typical position and relations of the centre supplying the branchial musculature, and therefore, strictly speaking, should not be regarded as part of the vagal lobe.

Fig. 14 is a section of the facial lobe of the Carp at the level of the origin of the glossopharyngeal nerves. The facial lobe is seen situated centrally and dorsally and on either side the trunks of the *communis facialis* are seen in section, giving off branches into the lobe.

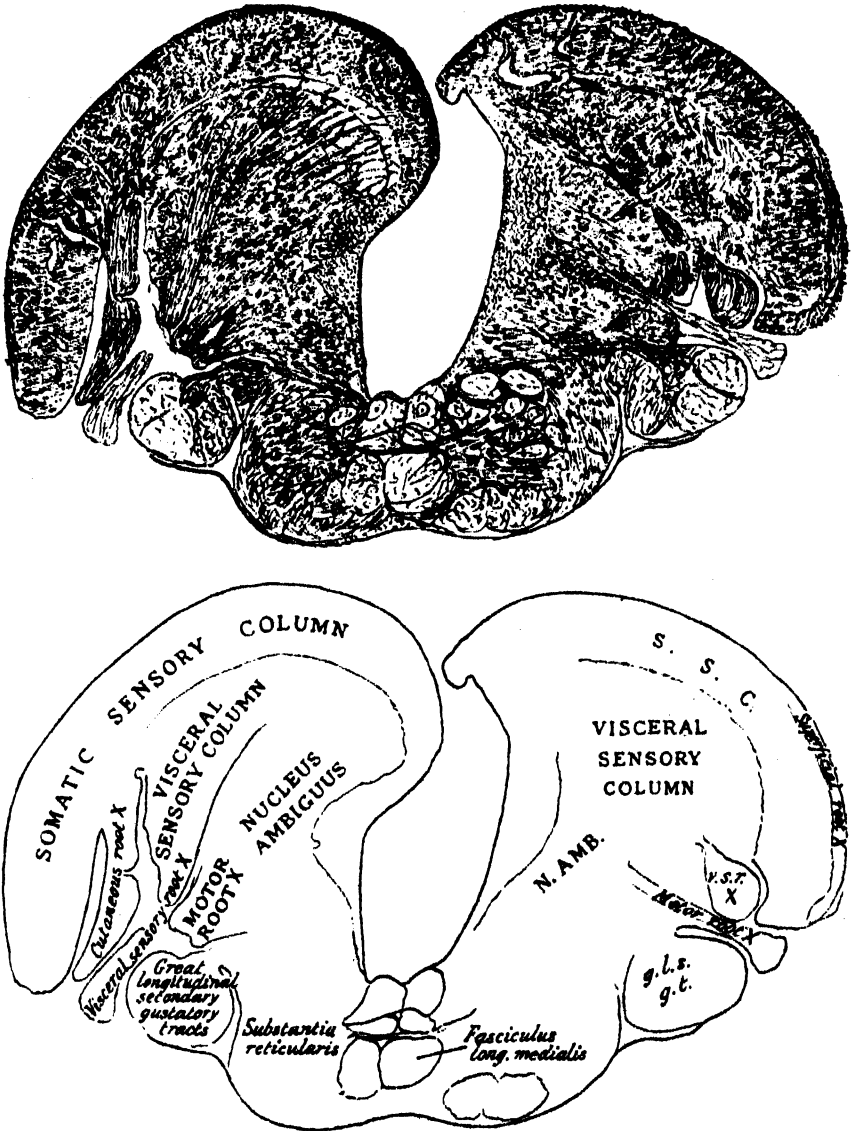


FIG. 13—Carp: Section across Vagal Lobes. *g.l.s.g.t.*, great longitudinal secondary gustatory tract.

Descending gustatory fibres are seen passing to the great longitudinal gustatory tracts, some fibres crossing to either side dorsal to the *fasciculus long. medialis*.

On the left side, fibres from the IXth nerve can be seen definitely entering the facial lobe ventral to the facial nerve trunk.

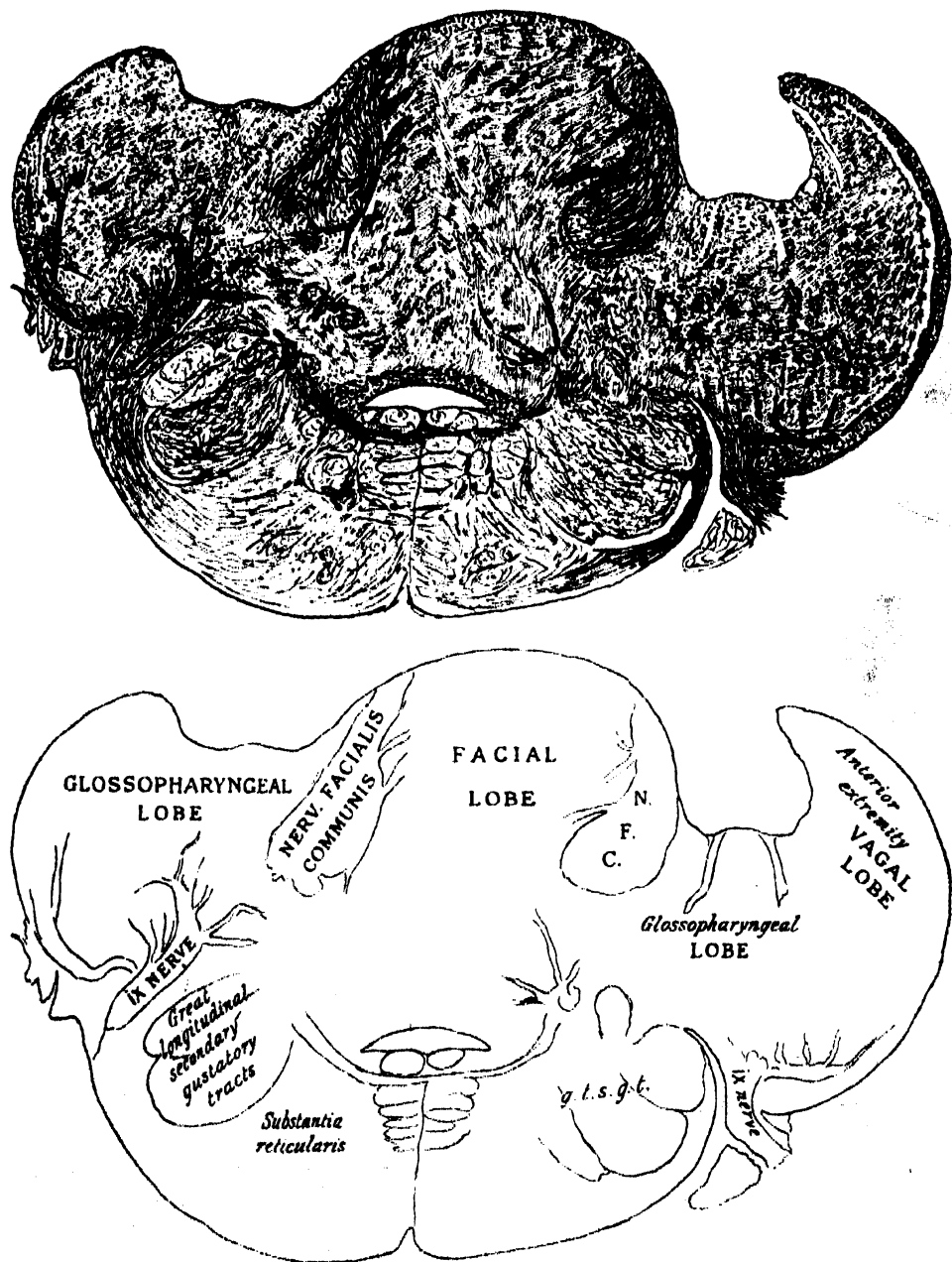


FIG. 14.—Carp: Section across posterior part of Facial Lobe.

We lay stress on this observation as this connection between the facial and glossopharyngeal does not appear to have been noted by previous writers.

lobe at the anterior end of the glossopharyngeal-vagal lobes, but the section reveals that in reality the facial lobe here occupies the whole dorsal half of the section, the ventricle lying centrally.

Roach.

This extension laterally of the facial lobe is limited to a very small depth, as the anterior ends of the glossopharyngeal soon appear on either side of the central prominence of the facial, and the facial lobe becomes limited to the medial area with the glossopharyngeal lobes on either side.

The next sections show a gradual diminution of the facial lobe and the glossopharyngeal lobes give off descending fibres, some of which enter the ascending gustatory tract and some cross over beneath the ventricle to the opposite side. The ventricle here widens out and soon there is seen a wide vertical space separating the vagal lobes. These lobes produce merely a slight bulging of the lateral walls of the *medulla*, and therefore the section still retains its oval form.

As regards its minute anatomy, it will be noticed that compared with the Carp the somatic sensory area of the vagal lobes is small in extent and still smaller is the *nucleus ambiguus*.

The characteristics of the group of sight-feeding Cyprinoids, of which the Roach is the type, is a small facial lobe which, however, is bigger than would appear from its surface view, and the comparative smallness of the vagal lobes.

The Microscopic Anatomy of the Hind Brain of the Gudgeon.

We have already seen that the facial lobe of the Gudgeon is large. Its anterior portion tapers to a point which lies beneath the cerebellum. As sections are traced caudally the lobe becomes heart-shaped in section, with a tendency to divide medially. Further back the facial nerves pierce the medulla laterally at the same level as the Vth nerve roots enter. The lobe widens out and extends across the whole width of the hind brain. The facial nerves approach the middle line and divide into two branches, one of which immediately enters the facial lobe and the other turns sharply backwards. The branch to the anterior portion of the lobe passes laterally and dorsally to the superficial part of the lobe; the posterior branch passes backwards and at a level corresponding to the anterior extremity of the vagal lobes merges with the ventral aspect of the facial lobe and supplies its posterior portion.

The above details are readily understood by a reference to the diagrams I to VI (fig. 18) and figs. 19 and 20.

The distribution of the facial nerve is markedly different from the condition found in the Roach and in the Carp.

The division of the nerve into two main trunks, one leading to the anterior portion and the other to the posterior portion of the facial lobe, may possibly be correlated with the presence of barbels in this fish and with its grubbing method of searching for food. It will be therefore important to enquire whether a similar condition occurs in the Barbel.

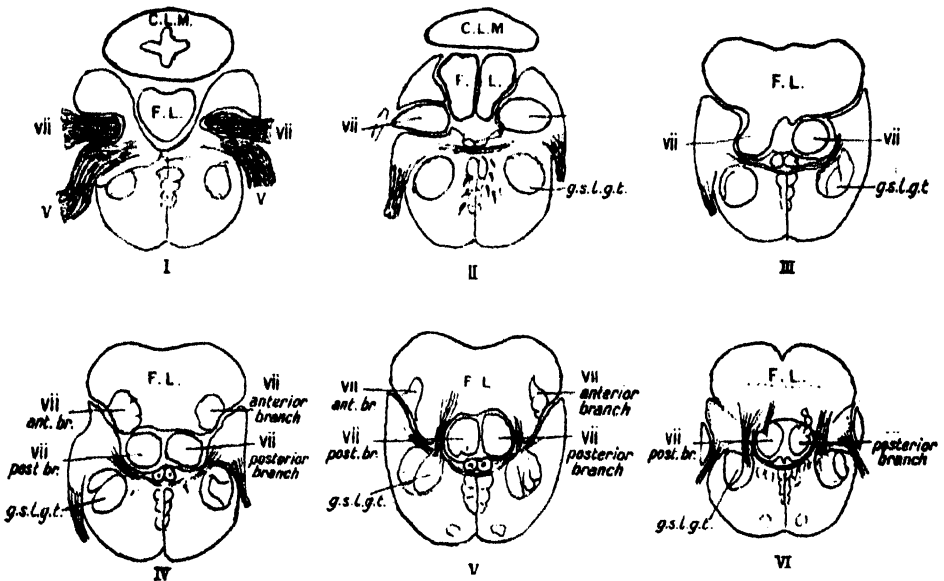


FIG. 18—Facial Lobe and Nerve of Gudgeon (i) to (vi) from before backwards. *ant. br.*, anterior branch; C.L.M., cerebellum; F.L., facial lobe; *g.s.l.g.t.*, great secondary longitudinal tract; *post. br.*, posterior branch.

The Facial Lobe of the Barbel.

We have seen in the naked-eye drawings of the Barbel the large size of the cerebellum and valvula. We have described the division of the facial nerve in the Gudgeon.

In the Barbel there is also a division of the facial nerve into an anterior and posterior portion, but the details differ widely from the condition found in the Gudgeon.

A study of the diagrams I to VIII (fig. 21) shows that the facial nerve after entering the *medulla* soon becomes club-shaped in section, the body of the club lying ventrally while the handle extends dorsally, and breaks up into a

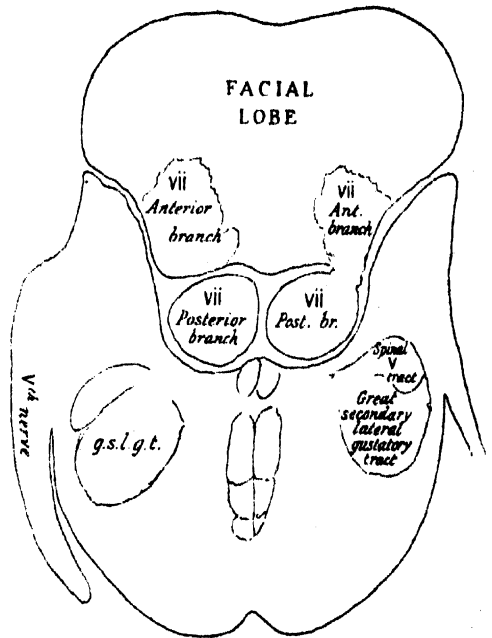
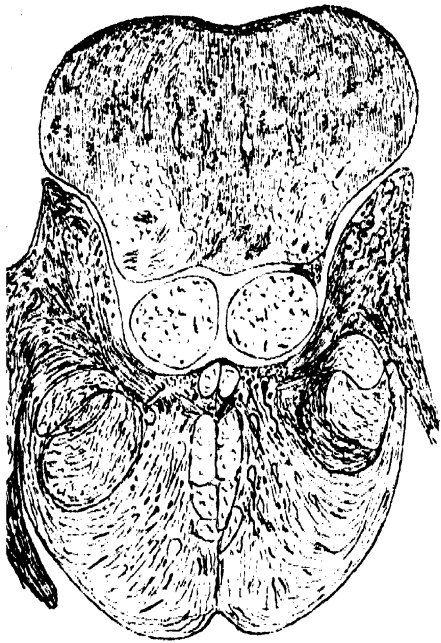


FIG. 19.—Gudgeon: Section across anterior portion of Facial Lobe.

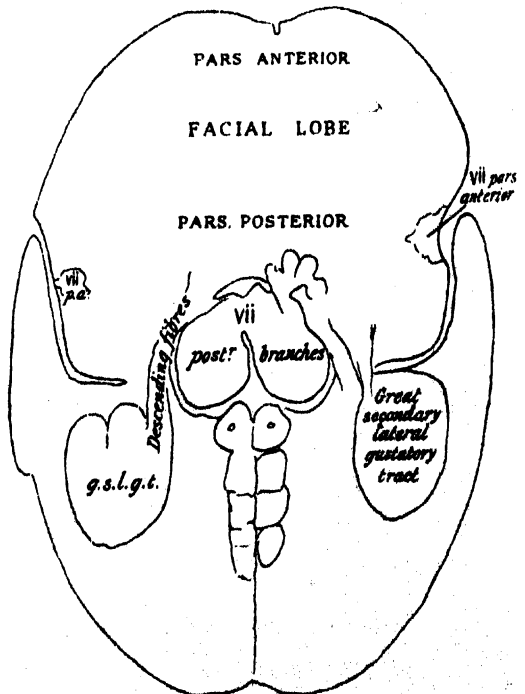
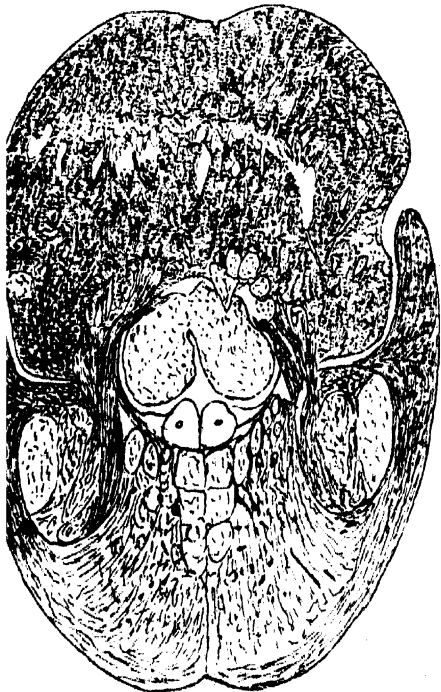


FIG. 20.—Gudgeon: Section across posterior portion of Facial Lobe.

number of small bundles of nerve fibres, which at first appear on the dorsal margin of the small anterior portion of the facial lobe, and terminate in the central portion of the lobe which may be designated the *pars anterior*. The larger part of the facial nerve then passes backwards and appears at first as two columns on either side of the facial lobe, which now appears heart-shaped in section; these columns lie towards the apex of the lobe which points ventrally (IV and V). As the facial lobe enlarges and extends deeper into the *medulla* the facial nerve becomes spread out into two crescentic areas embracing the lateral margins of the lobe (VII).

From the concavity of the crescent branches are then given off and enter the lobe; the last portion of the posterior trunk of the facial to break up into branches is that lying along the ventral margin of the lobe. The facial lobe then appears in the sections as an egg-shaped area lying in a cup formed by the vagal lobes and finally takes on an inverted heart-shape before it tapers to a blunt posterior extremity.

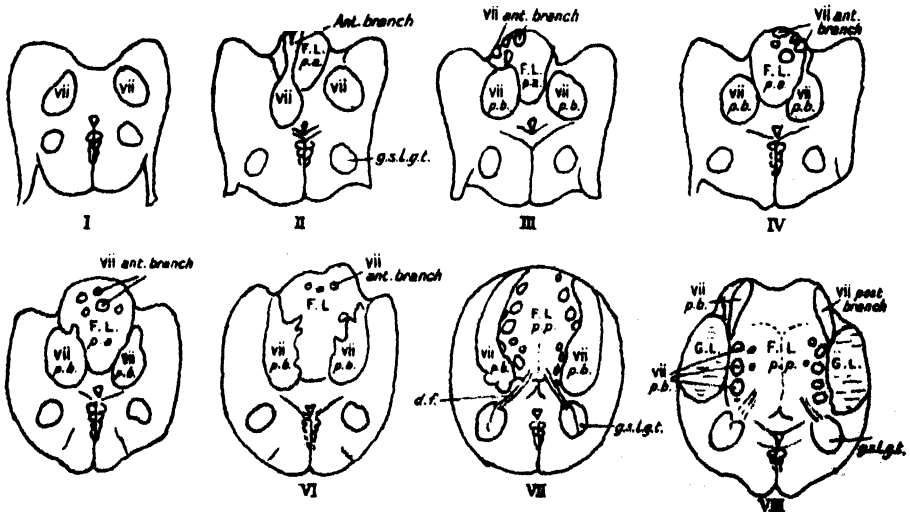


FIG. 21—Facial Lobe and Nerve of Barbel. *vii*, facial nerve; *vii p.b.*, facial nerve, posterior branch; *F.L.p.p.*, facial lobe, pars posterior.

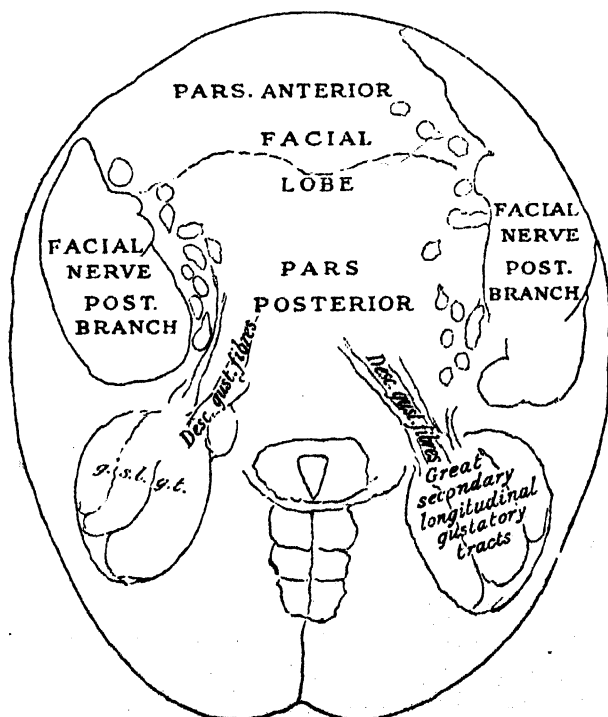
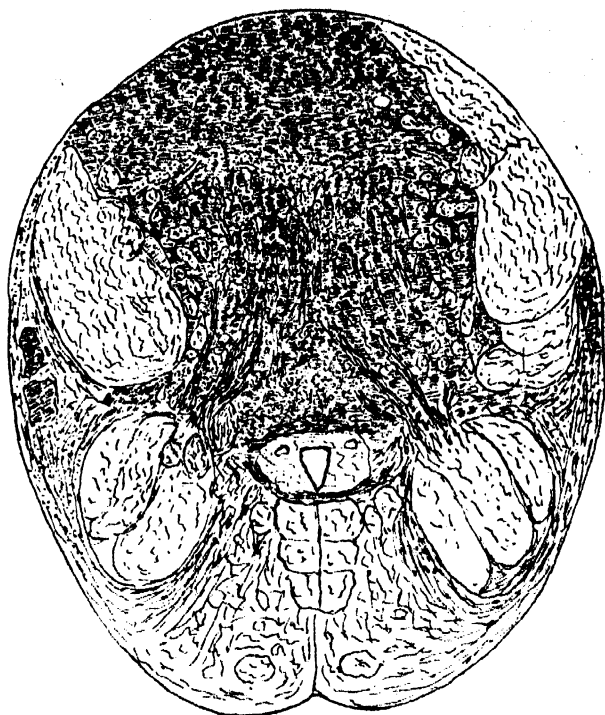


FIG. 22—Barbel: anterior to vagal lobes. *f.n. post. br.*, facial nerve posterior branch.

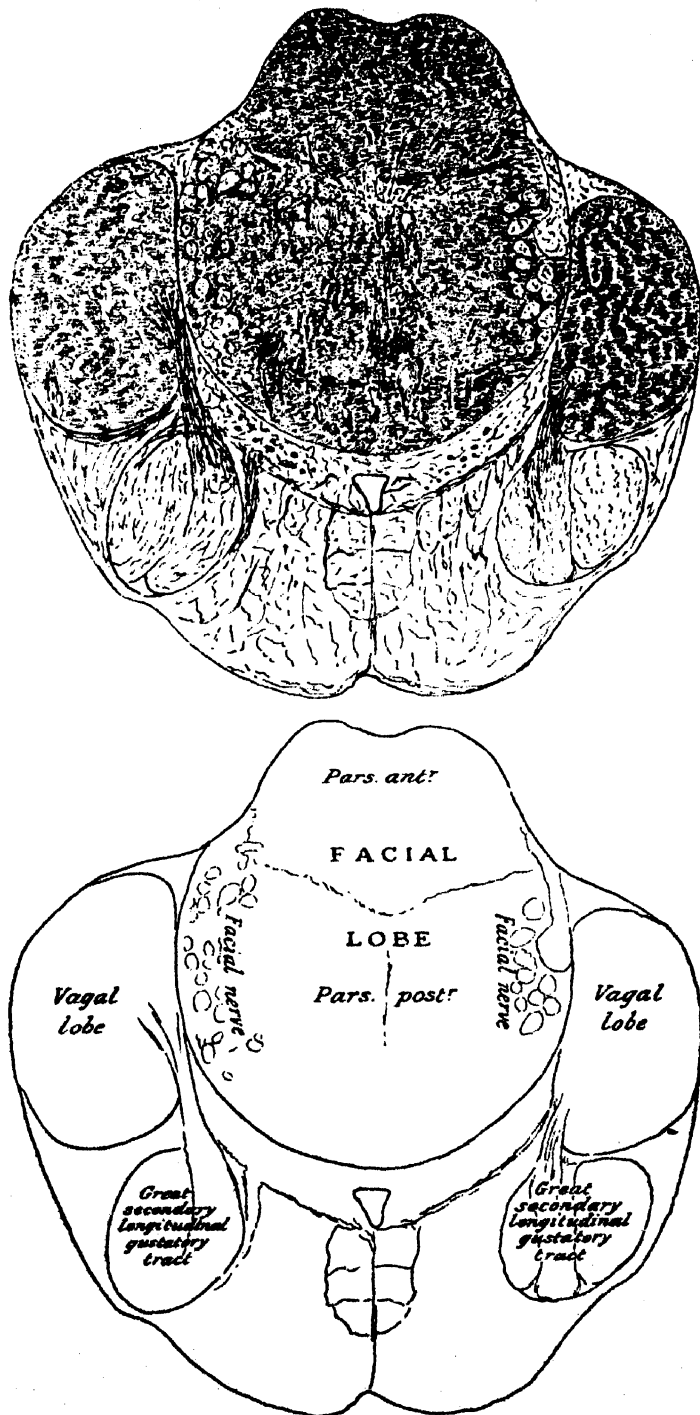


FIG. 23—Barbel: level of vagal lobes.

The Facial Lobe of the Tench.

The hind-brain of the Tench is remarkable on account of the fact that the vagal lobes are similar in type to that found in the Carp and that the facial lobes have a tendency towards the type found in the Barbel and the Gudgeon.

A study of the diagrams I to IV (fig. 24) will make this statement clear ; in fig. 24, I, and fig. 25 the facial lobe is seen with the facial nerve, giving

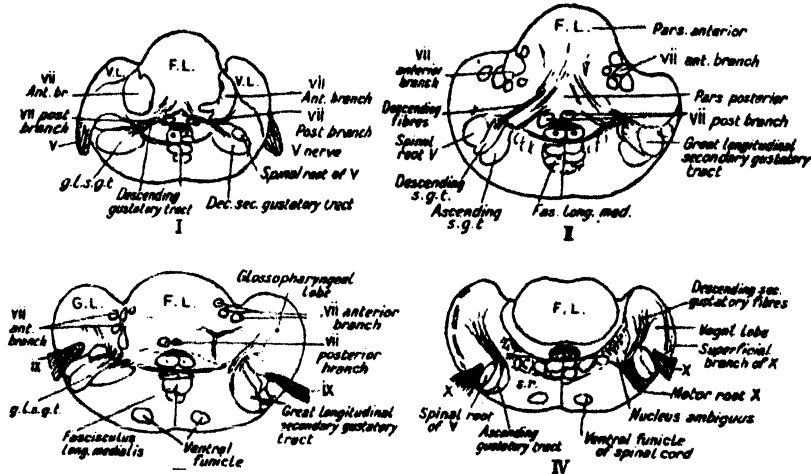


FIG. 24.—Facial Lobe of Tench.

off branches from its inner margin to the anterior portion of the lobe. A small posterior branch is clearly indicated arising from the ventral extremity of the nerve on either side. These small columns lie immediately dorsal to the ventricle, and are separated from the facial lobe by descending gustatory fibres passing down to the great longitudinal secondary gustatory tract. In diagrams II and III the main or anterior branch of the facial is seen to divide up into bundles at the lateral margin of the facial lobe on its dorsal aspect ; these bundles finally enter the substance of the lobe at the level of the appearance of the glossopharyngeal lobes, and tend to separate the latter from the facial lobe.

In the diagram II and in the drawing, fig. 26, the posterior branch breaks up into fibres supplying a small central triangular area bounded by descending fibres from the main body of the facial lobe.

In diagram IV a section is shown which is very similar to that of a section across the vagal lobes of the Carp, although the size of the lobes are smaller and do not present the same wing-like appearance. In diagram III on the

right communicating fibres are seen passing from the facial to the glosso-pharyngeal lobe.

The above descriptions of the facial lobe and the facial nerve in the Gudgeon, the Barbel and the Tench suggest that the existence of a divided facial nerve in these fishes may be associated with the presence of barbels and their peculiar grubbing habit of feeding.

The Barbel and the Gudgeon grope and grub in the gravel in search of food ; in the former the posterior division of the facial nerve is very marked and the *pars posterior* much larger than the *pars anterior*, in the latter the division of the facial nerve is into branches of nearly the same size, and the areas of the facial lobe supplied by the two branches occupy an equal area. The Tench, on the other hand, is characterised by a large branch which supplies the greater part of the facial lobe and a very small branch supplying a minute area of the lobe.

It would appear, therefore, that in those fish in which the grubbing habit is marked, there is a great development of the posterior part of the facial lobe ; on the other hand, in the Tench with slight grubbing habits the posterior branch of the facial is rudimentary and the area supplied by it is vestigial.

The great size of the vagal lobes in the Tench is clearly due to its present habits of feeding which are those of a mud-eater.

I wish to thank Mr. B. G. Clarke for his assistance in the preparation of the sections and also Mr. R. H. Burne for help in supplying me with the literature.

Dr. Tate Regan in his book on British freshwater fishes gives a synopsis, based on external characters only, of the British species of Cyprinoids.

In the preceding study of the configuration of the hind brain, I have been able to divide those members of this family, that I have so far examined, into three groups, each group having a particular method of feeding associated with a characteristic picture of the *medulla oblongata*.

It is interesting to note how his groups, based on their external characters, seem to conform with my grouping.

(I) of 'Tate Regan's Synopsis' (p. 169). Dorsal fin long, anal fin short ; last simple ray of each more or less distinctly spinous and serrated, contains : A, the Carp, with two barbels ; B, the Crucian Carp and the Goldfish, no barbels.

These three, we have seen, possess enormous vagal lobes, and are "mud-eaters."

(III) Dorsal fin short, anal fin long with 15 to 29 branched rays, with a keel behind the pelvic fins over which the scales do not pass.

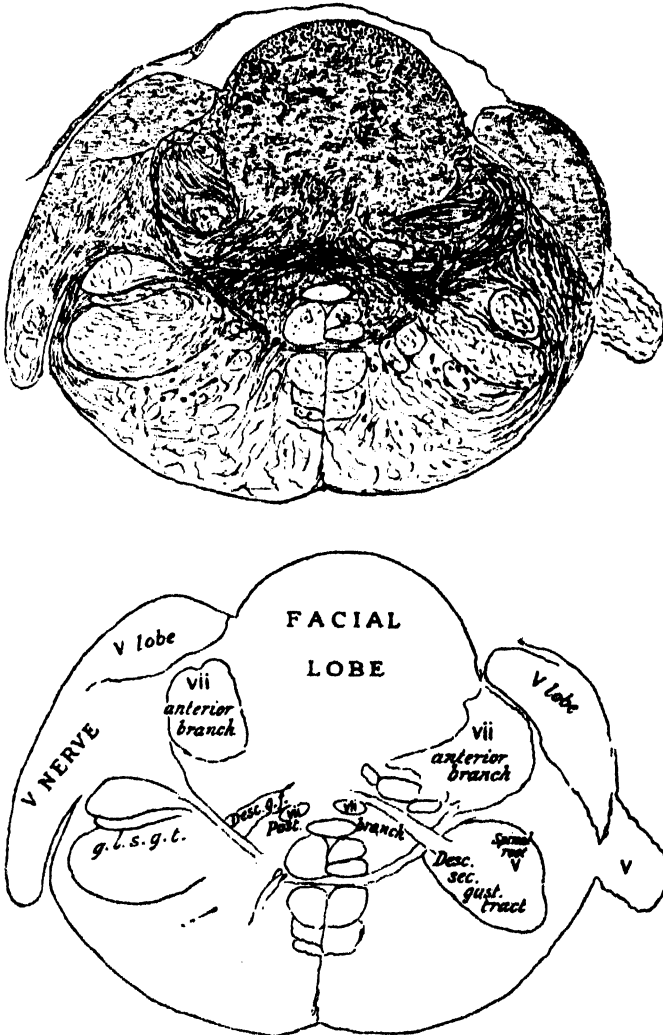


FIG. 25—Tench.

Contains A, the Bream (Common) or Carp Bream. This fish is also a mud-eater and the vagal lobes are large.

The Tench, we have already seen, should be put with the Barbel and the Gudgeon, although its vagal lobes are large, on account of the two divisions of the facial nerve, and the large facial lobe.

(II) of '*Tate Regan's Synopsis.*' Dorsal and anal fins short or of moderate length, neither with more than 13 branched rays.

A. *Mouth with Barbels.*—Includes the Barbel, the Gudgeon and the Tench.

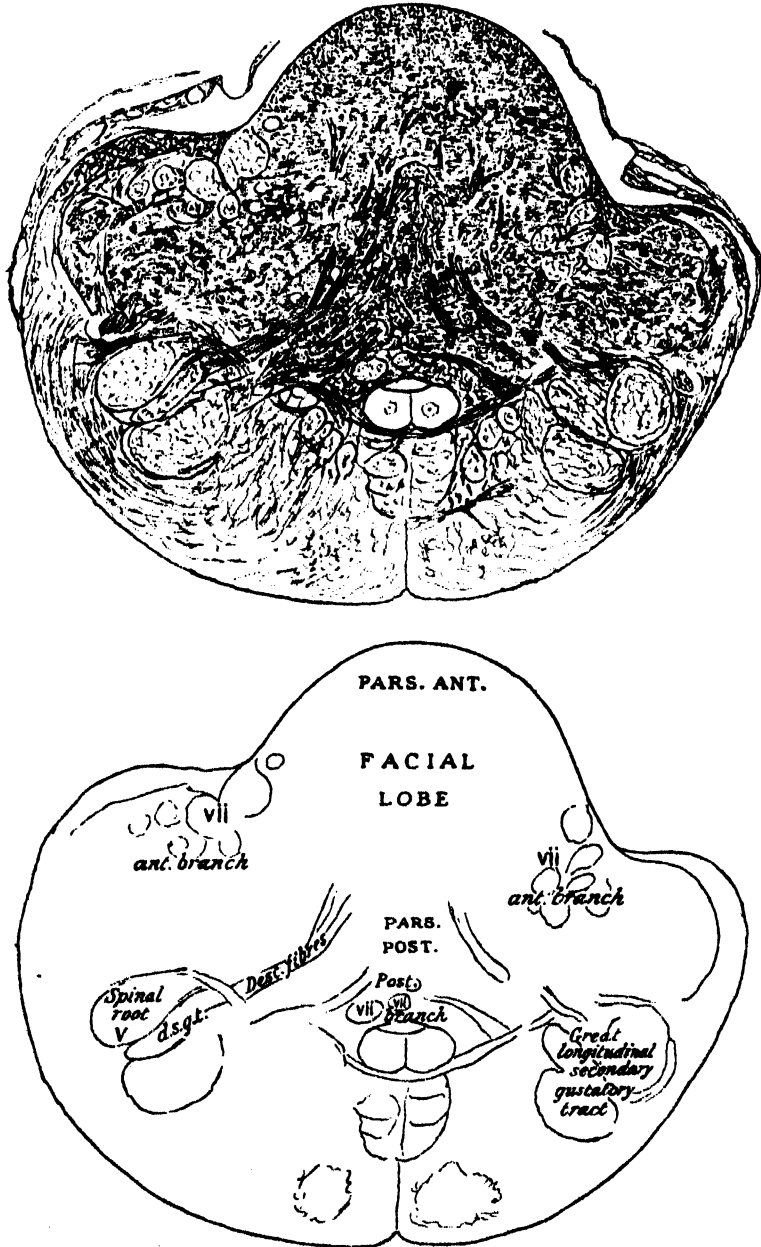


FIG. 26—Tench.

These are in my third group, and are characterised by a large facial lobe, a divided facial nerve, and the first two by their grubbing habits of feeding.

B. No Barbels.—Includes the Chub, the Dace, the Roach, and the Rudd.

All four have minute facial lobes, small vagal lobes, and all are active fish, which will take a fly, and feed mainly by sight.

So that with the exception of the Bream, which belongs to (III) and must be put with the Carps (I), the external characteristics correspond exactly with my classification.

I—With my group I.

II—A, with my group III.

II—B, with my group II.

It is certainly a very instructive fact that external characters, type of hind brain and habits of feeding should so closely agree.

It was hoped when this research was commenced, that some light would be thrown on the significance of the central facial lobe of Cyprinoids and Cobitidæ. But, as far as my investigations have gone, no solution of the problem has arisen. The large size of the facial lobe in the Barbel and the Gudgeon has been explained, and earlier work has elucidated the problem of the large vagals in the Carp and the Goldfish.

It is hoped that further work on the microscopic anatomy of the Minnow, the Bleak, the Bream, and the Loach may help to throw light on various points that arise from the observations already made. We believe, however, that this comparative study has revealed certain facts that are of biological importance.

[*Note added May 1st, 1931.*—Since the above was written I have examined microscopically serial sections of the hind brain of the Loach, *Nemachilus barbatulus*, and the Minnow. It is found that the facial lobe of the Loach is of the same type as is found in the Gudgeon, and the division of the facial nerve is very marked. The Minnow has a *medulla* of the same type as is found in Group II with slight minor changes in the facial lobe of no significance.]

Summary.

An examination of the brains of the Cyprinidæ of British waters with special reference to the conformation of the *medulla oblongata* leads to the conclusion that the Cyprinoids can be divided into three groups according to their type of hind brain and that these groups have similar habits of feeding and also present similar external characteristics.

The vagal lobes are large in the Carps, the Goldfish and the Bream and these are all mud feeders. Both the vagal lobes and the facial lobe are small in the

Roach, the Rudd, the Chub and the Dace, all of which take a fly and feed largely by sight.

The facial lobe is large in the Gudgeon, the Barbel and the Tench. The facial nerve in this group, the members of which possess barbels, divides into two branches after entering the *medulla*. These branches vary greatly in size; in the Gudgeon they are fairly equal in size, in the Barbel the anterior branch is small and the posterior large, while in the Tench the anterior is large and the posterior rudimentary.

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The Measurement of Actinic Erythema Produced by Ultra-Violet Radiation with Special Reference to the Latent Time.

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(Communicated by Sir Edward Sharpey-Schafer, F.R.S.—Received March 2, 1931.)

[PLATE 15A.]

§ 1. *Introduction.*

The quantitative study of the biological action of radiation is generally considered difficult in the case of skin erythema, and results obtained by different workers using ultra-violet radiation to produce the erythema are often contradictory. Much quantitative work has been done to ascertain how far any biological action runs parallel with the amount of physical stimulus, but this has usually been done on plants, histological sections of tumours, cultures, albumen, etc., and using more often γ -rays and X-rays than the longer ultra-violet rays.

The present paper is preliminary to further study, and is concerned with the precautions necessary to obtain reliable and consistent measurements of the amount of skin erythema resulting from exposure to ultra-violet rays.

§ 2. *Methods of Measuring Actinic Erythema.*

Fig. 1 serves to recall the four measurements which can be made in order to estimate and compare actinic erythemas. The observation of the depth of

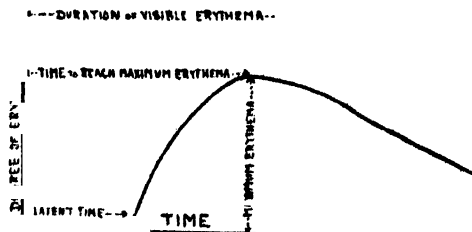


FIG. 1.—Method of Measuring Actinic Erythema.

erythema would include the observation of colour which will vary according to the quality of the radiation (Shattock and Waller, 1929 ; Uhlmann, 1930).

Some observers have attempted to estimate colour and depth of erythema by viewing the skin through coloured gelatin sheets (Hausser and Vahle, 1922), or by matching it to colours painted on a card. Wedges have also been suggested, but the tint varies with their thickness. I have found Lefranc's Table of Pastels with its great range of numbered tints useful. I also made up a series of the British Drug House capillary tubes as supplied for capillator use using phenol red p_H 6.8 to p_H 8.4 diluted to various amounts with a buffer solution of p_H 7.4. This standard is simple and can always be exactly reproduced. Excellent matches are obtainable and the depth of erythema is expressed by the number of the capillary, *i.e.*, by a known p_H concentration. The only disadvantage compared with pastels arises from the transparency of the capillary, and a constant method of looking at it relatively to the illumination of the room must be observed. In Germany more complicated methods of estimating the colour have been devised, *e.g.*, an apparatus based on Ostwald's colour triangle and a rotating sector in which any colour is expressed by two letters of the alphabet (Memmesheimer and Mathaei, 1930).

With regard to the depth of erythema, it is usual in England to speak of first, second and third degree erythemas which correspond roughly to slight, medium and severe erythemas. In Germany a great deal of attention has been devoted to devising methods of estimating the "rötungsgrad" and the instrument described by Schall and Alius (1925) claims to measure the degree of erythema by means of a scale divided into 30 units. They and others have published curves showing the relation between depth of erythema and exposure.

The observations of the time taken for the erythema to reach a maximum (which may vary from 7 to 12 hours); or of the duration of the visible erythema (ranging from 12 hours to several weeks) are valuable as confirmations of the conclusions reached by estimating the erythemas either by the maximum depth or by the latent time (see § 4 below). It is this last method of measuring erythemas which is the special study of the present paper.

§ 3. *Experimental Arrangements.*

Observations may be made statistically, *i.e.*, on a large number of persons and for certain purposes this may be desirable, but the conclusions based on few observations on each one of many subjects must be carefully scrutinised even where the conditions of irradiation are carefully controlled. For many purposes this method is unsuitable. It is necessary in order to obtain consistent results, to experiment on one subject, to irradiate very small areas not

usually exposed to light, and to make very frequent observations subsequently to the irradiation. This can only be done satisfactorily on oneself.

The experiments herein described were conducted with a quartz mercury lamp, the current through which was controlled by a rheostat. With such a lamp there is no complication due to sensible heating or reddening of the skin. The conditions of running remain practically constant, as shown by ammeter and voltmeter, after the necessary preliminary running of half-an-hour, and there is no complication as with a carbon arc lamp due to the intensity of radiation varying in different directions.

Fig. 2 (Plate 15A) which includes a centimetre scale gummed on the skin, is a photograph of a number of irradiated areas of skin taken about 4 hours after the exposures were made and shows the smallness of the areas (0.7 cm. diameter) exposed. The exposures were made on the right outer thigh 120 cm. away from the lamp (148 volts; 2.1 amps.). The exposures are increasing from left to right in the upper, and from right to left in the lower row of exposed

Table I.

Exposure in minutes	3	6	9	12	15
Latent time in hours	2½	2¼	1¾	1¼	1¼
Exposure in minutes	15	12	9	6	3
Latent time in hours	1½	2¼	2	2½	3¼

areas. The data relevant to this particular set of observations reading from left to right are given in Table I; their purpose was to determine what

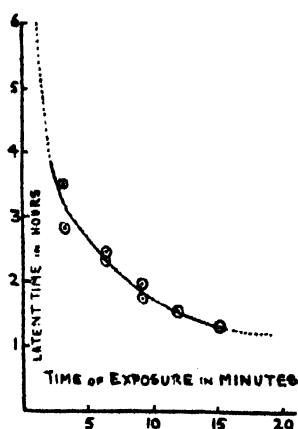


FIG. 3.

degree of accuracy one could hope to obtain in subsequent experiments. The result of plotting the latent times against the exposures appears on one curve, fig. 3. The exposures were made in the early part of the day and observations were made about every 10 minutes until the first appearance of the erythema, and many observations were made subsequently. Experience soon taught when the most frequent observations were necessary. In this particular experiment the faintest fifth erythema had already disappeared by the evening, the fourth had disappeared by midnight, the third was barely visible next morning, while the second and first erythemas persisted and merged into pigmentation, visible for about a fortnight.

§ 4. *The Latent Time.*

The relation between latent time and length of exposure has already been shown graphically in fig. 3. Further series of observations are given in figs. 4 and 5. It will be observed that exposures were made for varying distances. The relation between distance and the exposure necessary to produce a given erythema will be the subject of a subsequent paper.

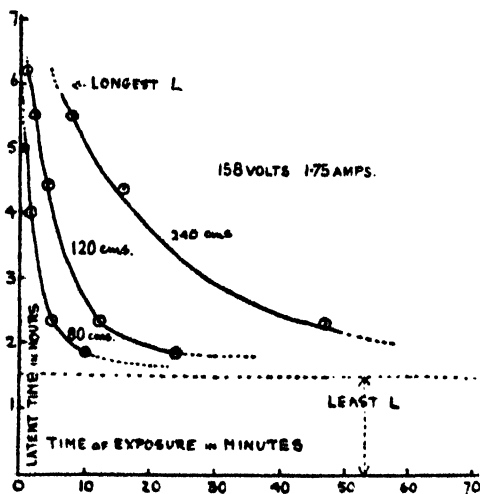


FIG. 4.

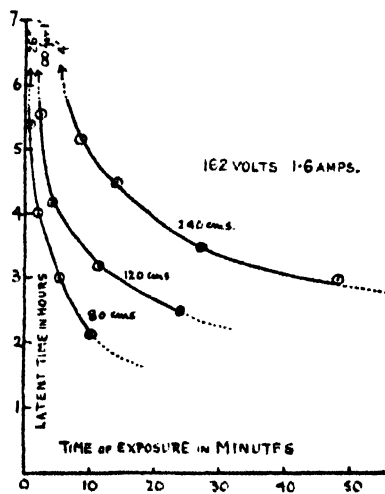


FIG. 5.

It will be noticed that the greatest latent time never exceeds $6\frac{1}{2}$ hours for the given subject (a few observations for short exposures do not appear on the curves as no erythemas were observed), and that this limit is the same for the shorter and longer distances. The least latent time from these and other observations for the given subjects is $1\frac{1}{2}$ hours.

The curves are very similar to those obtained by Rajewsky (1929) connecting the latent time before the occurrence of coagulation of albumen and the length of exposure to ultra-violet radiation.

It is interesting to compare these curves with those obtained for a photographic plate. Strips of Ilford Process plate were exposed for six different periods to the light of one candle placed 10 feet away from the plate. A small portion of each strip was left unexposed. The plate was then developed in hydroquinone developer and the latent time before the first appearance of any darkening in the exposed parts as compared with the unexposed part was noted by means of a stop watch. The exposures were varied from 3 seconds to 10 minutes, i.e., in the ratio of 1 : 200, and the variations of latent time with

length of exposure for one plate is shown graphically in fig. 6. The curve has the same general appearance as those shown in figs. 4 and 5, i.e., the latent

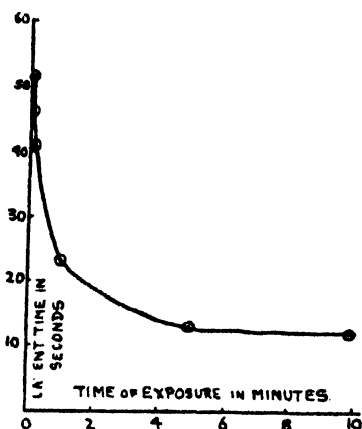


FIG. 6.

time increased very rapidly for short exposures and tends towards a least value for longer exposures. The actual figures of course, vary with the type of plate and the state of the developer. No simple formula for these curves is deducible nor is any advantage obtained from plotting reciprocal or logarithmic values of one or other of the ordinates.

§ 5. Conclusions.

The relation between the latent time before the appearance of actinic erythema and the time of exposure to a constant source of ultra-violet radiation, a mercury vapour lamp, held at a constant distance away from the skin is found to be a curve which terminates at a least exposure corresponding to a longest latent time. Beyond this the exposures are not sufficient to produce visible erythema. The latent time tends to a limiting value as the time of exposure is increased.

The results shown were obtained by making frequent observations on one day of numerous small areas of skin not usually exposed to light and on one subject. It would be expected that similar curves would be obtained with any other individual subject.

The results suggest that for scientific purposes the observation of the latent time provides the most accurate and simple way of estimating the effect of the ultra-violet radiation on the skin. No apparatus is necessary except a watch. Moreover the difficulty of comparing the results of one observer with those of another, when these are based on measurements of the degree of erythema, are obviated.

§ 6. Summary.

Consistent results regarding the biological action of ultra-violet radiation, as measured by the resulting skin erythema, can be obtained provided the precautions described in the paper are taken. The relation between the latent time (i.e., the time which elapses between exposure and the first visible signs of erythema) and the time of exposure to radiation of a constant intensity, is exhibited in graphical form. The curve terminates at a least exposure corre-

sponding to a longest latent time ; beyond this the exposures are not sufficient to produce visible erythema. The latent time tends to a limiting value as the time of exposure is increased. The curve is very similar to that which is obtained when the time of exposure to light of a photographic plate is plotted against the time which elapses before the first appearance of the image during development.

The latent time of erythema provides the most accurate and simple way of estimating the effect of ultra-violet radiation on the skin, and it is easily measured. Moreover the difficulty of comparing the results of one observer with those of another, when these are based on the measurement of the degree of erythema, are obviated. Observations of the degree and duration of erythema can confirm roughly the results obtained by measuring the latent time.

When it is desired to get the maximum contrasts due to differing conditions of radiation, exposures should be chosen which will lie on the steep part of the curve, corresponding to rapid variations of the latent time with exposure and to slight erythemas which will even visually differ one from another more than will deep erythemas.

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The Relation between Energy Doses of Ultra-Violet Radiation and Actinic Erythema Produced.

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(Communicated by Sir Edward Sharpey-Schafer.—Received March 2, 1931.)

[PLATE 15B]

§ 1. *Introduction.*

The purpose of the investigation was to ascertain what relation exists between the effect of radiation from a mercury vapour lamp on the skin, as measured by the visible actinic erythema, and the total amount of radiation energy or "dose" received.

An international unit of intensity for ultra-violet radiation is under discussion. Whether a physical unit or a biological one based on erythema of the skin be finally adopted, erythema will always remain one of the most important phenomena occurring in actinotherapy and one which gives some direct information regarding the response of an individual to radiation.

It is often assumed that an alteration in the intensity of ultra-violet radiation will not alter the degree of erythema, provided the same "dose" be given. No such *a priori* assumption is possible regarding the relation to be expected between the degree of biological action and the intensity of ultra-violet radiation. Indeed the amount of each specific biological reaction resulting from any specific kind of radiation— γ -rays, X-rays, or ultra-violet rays, must be studied separately as regards determining whether, when the intensity of irradiation varies and the dose (intensity $\times$ times) is kept constant, the amount of biological action is the same.

Liechti (1929) gives an interesting tabulated summary and full references of the results of some 21 experimenters, in which the type of radiation, the object radiated and the "time factor" are collected. For a photo-chemical reaction in which the Bunsen-Roscoe law holds, the product of the intensity of radiation and the time of exposure is a constant. A time factor equal to unity corresponds to the above relation, a time factor less or greater than unity indicates that for weaker intensities of radiation the time of exposure must be increased or diminished correspondingly. The constant is less in

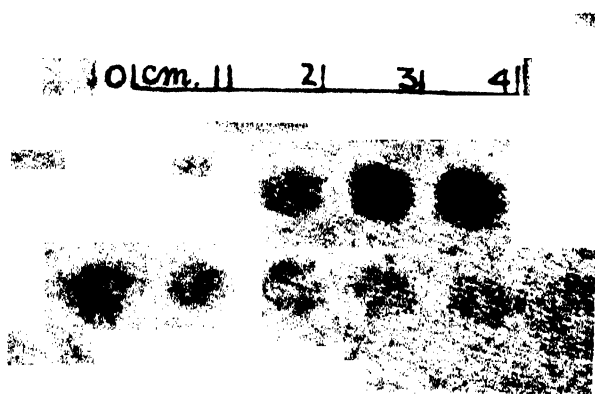


FIG. 2 (somewhat enlarged).

Distances. : Exposure
3 9



FIG. 1.

12, equal in 5, greater in 6 of these experiments. The majority of the experiments were conducted with X-rays and of the three conducted on the skin the constant is less than unity. There is at the present time general agreement with regard to this fact (Canti, 1929).

With regard to the effect of ultra-violet radiation, Seide (1928) gives the constant for the rate of destruction of *Ascaris* eggs as unity, a recent paper by Gentner and Schwerin (1930) gives a constant of 0.9 for the rate of coagulation of albumen, while Perthes (1924) and Freund (1920) give a constant of less than unity for skin erythema.

Angus's (1927) conclusions regarding skin erythema would correspond to a time constant of unity. He used an iron cored carbon arc and made exposures on a boy's chest varying the distances between 37 and 98 cm. A Griffith photo-electric cell was used for measuring the intensity of the radiation. H. J. Taylor (1929) on the other hand, using a mercury vapour lamp at distances varying from 20 cm. to 100 cm. and measuring the intensity of radiation by means of a thermopile, gives conclusions corresponding to a time constant of less than unity.

In view of these conflicting results, it seemed desirable to undertake a careful investigation over a large range of intensities of irradiation taking the precautions which have been advocated in the preceding paper to measure the effect of the ultra-violet radiation and paying very particular attention to the way the intensity of the radiation will vary with distance. The paper also shows the relation connecting dose and latent time.

§ 2. *Experimental Arrangements.*

The experimental arrangements are fully described in § 2 and § 3 of the preceding paper.

In the first series of observations (made in December, 1929) a heavy rigid stand was set up in front of the lamp, holding a vertical screen of three-ply wood in which a circular aperture of diameter 1.2 cm. had been cut. The aperture was adjusted to the same level as the centre of the lamp. Positions for the stand were previously marked on the floor with the aid of a set-square and scale and the lamp was not moved during the experiments. The radiation fell normally on the skin.

The subject (M.D.W.) stood with the area to be irradiated against the aperture. It is possible, though somewhat trying, to stand thus without appreciable movement for as long as an hour. The exposures were made in the earlier part of the day and were adjusted to give faint erythemas of about

3 hours' latent time and 30 hours' duration. Slight erythemas can be compared more accurately than deep erythemas. The height of the subject was altered slightly by standing on one or more drawing boards on different days so that new areas were irradiated and a chart was kept recording the areas already experimented with. The exposures with this arrangement varied from $1\frac{1}{2}$ minutes to 54 minutes. Assuming the inverse square law the distances corresponding were varied from 40 cm. to 2.4 metres.

A second series of experiments was made in June, 1930; one experiment with the original arrangement, and the others including greater distances and longer exposures and an accordingly modified arrangement, in which the subject sat on a mattress which could be moved to different distances from the lamp which was held in a stand near the ground. Protection from reflected radiation was effected with curtains. The rigid stand had to be discarded and exposures were made through one to three adjacent small holes of diameter 1.1 cm. in a double sateen-black paper screen applied to the skin. Levels and distances were adjusted as accurately as possible by means of plumb lines and tape measures. Any errors due to the possible want of normality of the incident radiation would be small at long distances. It was found practicable to sit as long as 3 hours with this arrangement and to increase the distances up to 5.6 metres.

In a third series of observations areas of 0.7 cm. diameter were exposed (see Plate 15B), and the number of areas exposed on a single morning was increased to 15.

§ 3. *Law of Inverse Squares.*

For a point source, $I = P/D^2$, where I is the intensity of radiation at a distance D , away from a source whose radiating power is P . The mercury vapour arc being 7.5 cm. long, it was considered necessary to see whether this law could be assumed for the distances in question.

I found difficulty in using a Griffith photo-electric cell for the estimation of the intensity of radiation when this varies over wide limits and to compare with certainty very rapid and very slow discharges of the electroscope. For short distances it seems impossible to avoid some pin-hole camera effect on the photo-electric surface and the condition of illumination of the surface was not the same as at greater distances even when the same diaphragm was retained and the high intensity radiations reaching the cell were reduced by means of filters.

The following photographic method is both simple and accurate and in my

opinion to be preferred. I have found that P.O.P. (Seltona) paper is visibly about four times more sensitive to variations in length of exposure than is the skin. A piece of paper in which 21 holes had been made was pinned on a drawing board over a piece of the P.O.P. paper. The board was placed at three different distances from the lamp, and for each distance a series of exposures was given by appropriately shielding or exposing the holes, see Table I. The exposures were chosen so that the "dose" (intensity $\times$ time

Table I.

Distances.

Exposures in minutes and seconds.

	cm.	' "	' "	' "	' "	' "	' "	' "
	120	2 15	3 22.5	4 30	5 37.5	6 45	7 52	9 0
	80	1 0	1 30	2 0	2 30	3 0	3 30	4 0
	40	0 15	0 22.5	0 30	0 37.5	0 45	0 52.5	1 0

of exposure) in any one vertical column would, if the inverse square law holds, be the same, and therefore the resulting darkening the same. By reference to the figure on Plate 15B it will be seen that the images are visually equal for the distances 80 cm. and 120 cm. and would be, *a fortiori*, equal for greater distances; while for the smaller distance, 40 cm., there is but a slight departure from equality. The inverse square law will be assumed in what follows.*

§ 4. Results and Conclusions.

First and Second Series of Experiments.—A large number of data were obtained in the first and second series of experiments the results of which will be summarised briefly, while the results of the final set of experiments in which more data were obtained on any one day will be exhibited in graphical form.

In the first and second series one day's observation consisted in giving equal "doses" assuming the inverse square law at three distances, for example:—

Table II.

Distances in cm.	Exposure in minutes.	Latent time in hours.	Erythema a maximum.	Comparison of erythemas.	Duration.	Amps.	Volts.
40	1.5	3	1st evening	} Equal	Just visible next morning	1.75	152
80	6	3	"			to	to
120	13.5	3	"			1.7	160

* Two large photographic firms assure me that Seltona paper used under these conditions follows the Bunsen-Roscoe law; moreover I verified this using a Pointolite lamp.

Comparisons will be based upon the observations of the latent times, the degrees and the durations of erythema being used as confirmatory evidence. It has been shown in the preceding paper that this is the most accurate measurement to make. The latent time is estimated to within a quarter of an hour. The conclusions to be drawn depend, of course, on the number of the experiments and whether any consistent tendency for the latent time to increase or decrease is apparent. The conclusions were made for each day separately, as even with rheostat adjustment the lamp does not burn exactly the same way from day to day. The distances were increased up to 5.64 metres; for such distances, in order to keep the length of exposure within practical limits, the aim was to produce very long latent times (6 hours) corresponding to very faint erythemas (already fading in the evening and no longer visible the next morning). The times are measured from the middle of the exposures—for example, with distance 80, 120, 564 cm. the exposures given were 2.3, 5.2 and 120 minutes respectively and resulted in visually equal, very slight, erythemas which appeared only after 6 hours and had faded by the next morning. The conclusions reached from 12 days of experiments is that the time factor is unity.

Third Series.—The most striking way of exhibiting the relation between dose and resulting erythema, provided enough data are available from a single day's observations, is by means of a graph. The data already exhibited in graphical form in fig. 4 of the preceding paper have been taken and the result of plotting the dose = intensity of radiation $\times$ time, against the latent time is shown in fig. 2. Fig. 3 has been similarly obtained from fig. 5 of the pre-

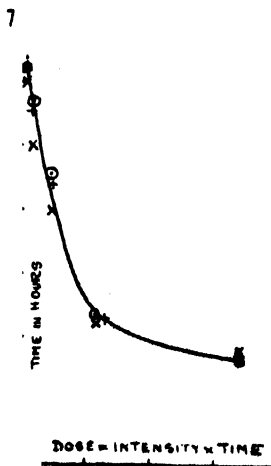


FIG. 2.

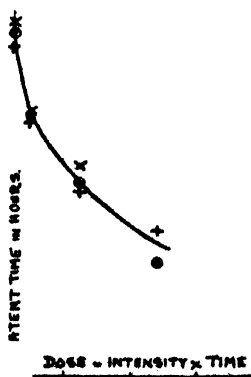


FIG. 3.

ceding paper. It will be observed that in both figures the three curves lie on a single curve and confirm the conclusion already reached, that, provided the total quantity of radiation or "dose" is the same, the biological action on the skin as measured by the latent time for the production of visible actinic erythema is the same.

In the above experiments the distances have been varied over very wide limits (40 cm. to 5.6 metres) corresponding to very great variations of intensity of radiation (200 : 1), and we conclude that for ultra-violet radiation and resulting skin erythema the time factor is unity. Finally it may be emphasised that whereas figs. 4 and 5 of the preceding paper give the relation between time of exposure and latent time, figs. 2 and 3 of the present paper shows the relation connecting dose and latent time.

§ 5. *Summary.*

The purpose of the investigation was to ascertain what relation exists between the effect of radiation from a mercury vapour lamp on the skin as measured by the resulting erythema and the total amount of radiation energy or "dose" (intensity $\times$ time) received.

The paper also gives the relation connecting dose and latent time. The question is of both theoretical and practical importance. It is a particular case of the general problem of how the extent of a biological reaction depends upon the quantity of any kind of radiation (γ -, X- or ultra-violet rays) producing it.

Particular attention was paid to the question of how the intensity of the radiation decreases with the distance from the source. The intensities were varied over wide limits (200 : 1) corresponding to distances varying from 40 cm. to 5½ metres. The effect of a given "dose" of the weak intensity is just as great as that of the most powerful intensity used, and it is concluded that the production of erythema follows the Bunsen-Roscoe law for a photo-chemical action, i.e., the time factor is unity.

If the latent time be plotted against the "dose" made on one part of a subject with the source of radiation constant, but the intensity of radiation varying, the observations lie on a single curve, i.e., if we take a separate series of curves connecting latent time and time exposure as obtained in the previous paper and plot "dose" instead of time of exposure, the separate curves become one. This curve gives the relation between the biological reaction of the skin as measured by the latent time for the production of erythema and the quantity of radiation received.

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The Vital Staining of Normal and Malignant Cells. IV.—A Comparison of the Vital Staining of the Parenchyma Cells of the Liver with Acid and Basic Dyes.

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(From the Laboratories of the Imperial Cancer Research Fund.)

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[PLATE 16.]

1. *Introduction.*

The injection of an acid dyestuff such as trypan blue into the animal body is followed by the appearance of dye droplets in certain of the cells, particularly in the macrophages of the so-called reticulo-endothelial system, and the cells of the connective tissues, liver and kidney. Rarely is any dyestuff found in epidermal, gland or germ cells. With a basic dye, such as neutral red, droplets can be stained in almost all the cells of the body. There has been much discussion within recent years as to the cellular mechanism involved in vital staining with these two types of dyestuffs. It is generally agreed that in vital staining with acid dyes, the coloured droplets that appear in the cells are new formations due to the segregation of the dyestuff. Actual staining of preformed cell structures is rare, but it does sometimes occur. For example trypan blue stains the milky secretion in the mammary gland cells.

A far greater variety of cell inclusions are stainable by neutral red, including

cytoplasmic vacuoles, metaplastic bodies, mast cell granules, and acid dye droplets. Hence the view has been advanced that while vital staining with acid dyes results in the production of new formations, basic dyes stain pre-existing granules and vacuoles in the cytoplasm.

In a former paper (Ludford, 1930) I have discussed the various theories of vital staining with basic dyes and brought forward evidence based on the study of acinar cells of the pancreas to show that in vital staining with basic dyes a similar process of segregation occurs as with acid dyes.

The pancreas cell was chosen for my former work because it is particularly favourable for observing the relationship between neutral red droplets and the cytoplasmic organellæ. It has the disadvantage that it is unstained by acid dyes. The experiments here described were carried out with parenchyma cells of the liver, which are stainable intra vitally with both acid and basic dyes. They confirm the conclusions of my former paper that both acid and basic dyes are segregated by cells, and give rise to new cytoplasmic formations. The present communication is therefore submitted in order to show that the same principles of vital staining apply to cells of widely different functional activity, and it is not proposed to continue further investigation of this aspect of the problem as a part of this series of researches.

2. Vital Staining with Trypan Blue.

Nassonov (1926) was the first to suggest that the segregation of acid dye droplets in cells was brought about under the influence of the Golgi apparatus. He drew attention to the topographical relationship between the Golgi apparatus and trypan blue droplets in liver and kidney cells. In these cells I was able to demonstrate both the Golgi apparatus and trypan blue droplets at the same time. My preparations showed that trypan blue droplets originated in these cells in relationship with the apparatus (Ludford, 1928).

Although Makarov (1926), and Cramer and I (1926) found that the Golgi apparatus of the liver cell undergoes alteration in form with changes of the state of functional activity, yet in the majority of the cells of the liver of vertebrates it appears as a system of branched filaments bordering the intercellular bile capillaries. When a mouse is vitally stained with trypan blue most of the dye droplets of the liver cells are to be found in this area, a distribution which serves to map out the intercellular bile capillaries.

Trypan blue droplets do not form to any extent in the parenchyma cells of the liver of the mouse until dyestuff has been injected several times. In

my former paper I figured dye droplets in liver cells subsequent to three injections of 0.5 c.c. of a 1 per cent. solution of trypan blue.

Fig. 1, Plate 16, is a photomicrograph of a frozen section of the liver of a mouse which had received 1.0 c.c. of 1.0 per cent. solution of Grübler's trypan blue on the first, fourth, sixth and ninth days of the experiment. It was killed and the liver fixed on the tenth day. The dye droplets are seen in the figure at the margin of the cells, bordering the intercellular bile capillaries.

3. *Vital Staining with Vital New Red.*

Vital new red (Grübler) is a good acid dye for vital staining. It is relatively non-toxic, but I have been unable to retain the dye granules in section after formalin fixation. Tissues of vitally stained animals were therefore examined *supra* vitally in Ringer solution. After eight injections of 0.5 c.c. of a 0.5 per cent. solution given during the course of 9 days, only a very few dye droplets were found in parenchyma cells of the liver. More dye was seen in these cells after 17 injections of 0.5 c.c. of the dyestuff, given over a period of 21 days. The dye droplets were far fewer in number than after staining with trypan blue, but they showed the same localisation in the cytoplasm.

4. *Vital Staining with Carmine.*

Carmine is a less diffusible dyestuff than trypan blue or vital new red, and for vital staining should be injected intravenously. In my experiments I used lithium carmine prepared according to the method of Kiyono. Before use it was sterilised by passing through a Berkefeld filter. This stock solution was badly tolerated by the mice, so before injecting it was diluted with sterile distilled water, the strength being increased with successive injections. The following injections were made intravenously into the tail vein :—

1st day—0.3 c.c. of 1 part carmine, 4 parts water.

3rd day—0.3 c.c. of 1 part carmine, 3 parts water.

4th day—0.4 c.c. of same dilution.

5th day—0.4 c.c. of same dilution.

6th day—0.5 c.c. of same dilution.

7th day—0.3 c.c. of equal parts of carmine and water.

8th day—0.3 c.c. of same dilution.

10th day—0.3 c.c. of same dilution.

Mice were killed 4 hours after the last injection. Pieces of liver were fixed in formol salt and frozen sections were stained with Harris's hæmatoxylin.

Preparations showed the Kupfer cells heavily laden with carmine, but relatively little was seen in the parenchyma cells of the liver. The dyestuff present, however, showed the same distribution as the droplets formed after vital staining with trypan blue.

5. *Vital Staining with Eosin and Orange G.*

Unlike the dyes to which reference has already been made, eosin and orange G on injection into the animal body are not segregated in droplets. Eosin diffuses nearly twice, and orange G approximately three times as fast as trypan blue in a 10 per cent. gelatine gel. Eosin is lipid soluble, but orange G is not. In my tests with these dyes 0.5 c.c. of 0.5 per cent. solutions were injected daily for 4 days, on the fifth day 1.0 c.c. was injected. Mice were killed, and the liver cells examined in Ringer solution 2 hours after the last injection. The parenchyma cells appeared to be diffusely stained. There were no dye droplets to be seen, either in the parenchyma cells, or even in the Kupfer cells. The latter were intensely stained by the other acid dyestuffs employed.

6. *Vital Staining with Neutral Red.*

Michaelis (1900) showed that after the injection of the basic dye neutral red, dye droplets appeared around the periphery of the parenchyma cells of the liver. More recently Dornesco (1930) has described the distribution of neutral red droplets between the nucleus and intercellular bile capillaries. Following the intraperitoneal injection of 1 c.c. of 0.5 per cent. solution of this dye into the mouse the liver cells are intensely stained in less than an hour. It is possible to make permanent preparations of the vitally stained liver by the method of Gardner described in my previous paper (Ludford, 1930). Fig. 2, Plate 16, is a photomicrograph taken from such a preparation. It shows the dye droplets localised in the same area of the cells as in vital staining with trypan blue.

7. *The "Krinom."*

In my previous paper reference was made to the granules formed in cells after intense staining with neutral red, which Chlopin calls the "Krinom," and believes to be of a protein nature. He has recently (1930) brought forward evidence to show that the "Krinom" formed as the result of vital staining with acid dyes is a basic protein, since it stains in sections with acid dyes, while the "Krinom" resulting from vital staining with basic dyes is to be regarded as an acid protein. Chlopin has figured the "Krinom" resulting from vital staining with neutral red in liver cells of the frog as irregular shaped

granules grouped around intercellular bile capillaries, in the same area of the cell as the Golgi apparatus is located.

I find exactly the same arrangement of the "Krinom" in liver cells of the mouse. My preparations were made from the livers of mice which had received three daily injections of 0.5 c.c. of an 0.5 per cent. solution of neutral red (Grübler) and one injection of 1.0 c.c. Mice were killed 6 hours later, and small pieces of liver were fixed in Helly's fluid, and sections were stained with eosin-azure. The "Krinom" stains blue against the pink background of the cytoplasm, and such preparations resemble those obtained by counterstaining with a red dye sections of the liver vitally stained with trypan blue, since the granules in both cases occur in the same region of the parenchyma cells. Fig. 3, Plate 16, shows the arrangement of the "Krinom" granulation in relation to the intercellular bile capillaries.

8. *Vital Staining with Brilliant Cresyl Blue and Toluidin Blue.*

According to the determinations of von Möllendorff (1921), brilliant cresyl blue diffuses more rapidly, and toluidin blue more slowly than neutral red. All are lipoid soluble basic dyestuffs, but are readily flocculated in contact with acid colloids.

Mice were vitally stained with brilliant cresyl blue (Grübler) and toluidin blue (Grübler) by injecting 0.5 c.c. of 0.5 per cent. solutions subcutaneously, and also intraperitoneally. Thin slices of liver were examined in Ringer solution at intervals after the injections. With both dyestuffs purple granules of varying sizes made their appearance in the same region of the parenchyma cells.

9. *Vital Staining with Methyl Violet 6B and Rhodamin B.*

Both of these basic dyestuffs diffuse more rapidly than neutral red; but rhodamin B is not flocculated by acid colloids at all, and methyl violet 6B is only flocculated to a negligible extent. They are both soluble in lipins. When injected into mice in 0.5 per cent. solution they failed to stain any granules in the parenchyma cells, but appeared to stain the cytoplasm diffusely. After five daily injections of 0.5 c.c. of an 0.4 per cent. solution of rhodamin B no dye droplets could be seen in the liver cells.

10. *The Nature of the Vitally Stained Granules.*

The experiments described in the preceding paragraphs have shown that there are both acid and basic dyes which are capable of inducing the formation

of coloured droplets in parenchyma cells of the liver. With both classes of dyestuff the droplets collect around the margins of the cell, bordering the bile capillaries. The chief distinction between acid and basic dyes is the longer time taken to produce a granular staining with an acid dye such as trypan blue than with a basic one such as neutral red. Twenty minutes after the intraperitoneal injection of 1 c.c. of a 0.5 per cent. solution of neutral red, liver cells are intensely stained. Two hours after the injection of the same amount of a 0.5 per cent. solution of trypan blue, no dye droplets are visible. After 24 hours only a very few droplets can be detected. The problem arises here as to whether basic dyes stain vacuoles present at the periphery of the liver cell, or whether the dye droplets are new formations, as are those which originate in vital staining with trypan blue. In a previous paper I have figured dye droplets in association with the Golgi apparatus of the liver cell in mice vitally stained with trypan blue. We have conclusive evidence in such a case that the dye droplets are new formations. In order to investigate whether basic dyes induce a similar formation of droplets, mice were vitally stained by injecting 1.0 c.c. of an 0.5 per cent. solution of neutral red intraperitoneally, and osmic impregnation preparations made of the liver after various intervals of time. Small pieces of liver were fixed in Champy's fluid 25 minutes, 1 hour, 4 hours, 7 hours and 24 hours respectively after injection of the dye. The tissues remained in the fixative 24 hours and were then washed in running water for another day, then in distilled water, repeatedly changed for a further 6 hours. They were next transferred to 2 per cent. osmic acid in an incubator kept at 35° C. for 4 days, then washed in several changes of distilled water, and left overnight in distilled water kept at 35° C. to complete the reduction of the osmic acid. Sections were cut 3 μ in thickness, and were put into turpentine for an hour to remove the black fat droplets. Neutral red was employed for staining as described in my former paper. In sections of the liver fixed half-an-hour after injection of the dye numerous granules are to be seen in contact with the blackened Golgi apparatus. They present an appearance identical with that seen in figs. 16 to 19 of my former paper (Ludford, 1928). Granules are seen in the preparation of the livers fixed at later intervals, but they are fewer in number after an hour, and not discernible after 24 hours.

Similar preparations were made after vital staining with the basic dye, rhodamin B, and the acid dye, eosin. No such granular aggregations were seen as occur after staining with trypan blue, or neutral red, even after six daily injections of these dyestuffs.

When thin slices of mouse liver are examined microscopically in Ringer

solution some of the cells are seen to contain fine pale yellow granules, or droplets in the same area. These normally occurring granules are smaller than most of the droplets formed after the injection of neutral red or cresyl blue. They probably represent constituents of the bile in process of excretion. In sections of liver fixed in Champy's fluid similar granules can be stained by neutral red, but I have been unable to stain droplets formed as the result of vital staining with neutral red after the same fixation.

A striking confirmation of the new formation of droplets is afforded by comparing osmic impregnated preparations of the liver vitally stained with neutral red with those of controls. Small pieces of Champy fixed liver were treated as described above. Sections were bleached in hydrogen peroxide (1 part of H_2O_2 to 4 parts of 80 per cent. alcohol). They were stained with neutral red. Numerous granules were present in the vitally stained livers, which presented a similar appearance to those seen in fig. 2, Plate 16; while relatively very few occurred in the control livers. There is, therefore, little doubt that the droplets, which appear in the liver cells after the injection of either an acid dye, such as trypan blue, or a basic one, such as neutral red, are new formations induced by the segregating mechanism of the cell.

When the liver has been vitally stained by the intraperitoneal injection of neutral red, a uniform staining of the parenchyma cells has never been obtained in any of my experiments. While in some parts of the organ the cells are intensely stained, in others little or no dye droplets are seen. Similarly when osmic impregnated preparations are made of the liver a uniform impregnation throughout even a small fragment of tissue is extremely rare. The explanation of this variation most probably resides in the varying state of functional activity of the cells. In a former paper Cramer and I (1926) have drawn attention to the complexity of the functional activities of the liver cells, and have attempted to correlate the variations in the form of the Golgi apparatus with the state of activity of the parenchyma cells. The occurrence of granules in unstained liver cells mentioned above, and the formation of dye droplets after vital staining in the same region of the cells, suggests that the dyestuff which enters the cell follows the normal path of excretion. It is therefore only to be expected that the degree of vital staining will be dependent upon the state of functional activity of the cells.

11. *The Relation between the Physico-chemical Properties of Dyes and their Vital Staining Capacities.*

Although the capacity to induce the formation of dye deposits in the liver cells does not depend upon the acidic or basic character of the dyestuffs, it is clearly conditioned by certain of their properties. The rate of diffusion of the dye is obviously an important factor since it determines its accessibility to the cells. A highly colloidal dye such as carmine, or isamine blue, becomes deposited at the site of inoculation unless injected intravenously, while the rapidly diffusing dyes like eosin and orange G quickly spread throughout the body and are rapidly excreted by the kidneys. The best dyestuffs for vital staining, as Schulemann (1917) found, are those which have been called semi-colloidal and are of a medium degree of diffusibility such as trypan blue and neutral red.

Once a dyestuff has entered the cell in sufficient quantity, whether or not it will be segregated depends largely upon the extent to which it forms colloidal aggregates. According to von Möllendorff (1925), "die zur Granulabildung führende Konzentrierung ist höchstwahrscheinlich eine Dispersitätsverminderung, die durch Electrolyte hervorgerufen wird."

In the case of basic dyes, von Möllendorff found that lipid solubility played a fundamentally important part in determining their capacity for staining cytoplasmic granules and vacuoles. He found the best dyes for staining granules were those which were relatively slightly soluble in lipids, but were readily flocculated in the presence of acid colloids. High lipid solubility favoured diffuse staining of the cytoplasm. Rhodamin B is an example of this type of dye. Other characters of dyestuffs which influence their vital staining properties are the ease with which they undergo reduction, and their chemical composition in as far as it determines their physico-chemical and colloidal-chemical properties.

Summary.

(1) There are both acid and basic dyes which are capable of staining droplets in parenchyma cells of the liver intra vitally.

(2) The dye droplets which are new cytoplasmic formations are for the most part arranged around the margin of the cells, bordering the inter-cellular bile capillaries (Plate 16).

(3) The dyestuff, which is segregated to form these droplets, has followed the normal path of excretion in the cell, and the process is dependent upon its state of functional activity.

(4) The capacity of a dyestuff to induce the formation of droplets in the cell is dependent upon certain physico-chemical properties. The rate of diffusion determines the accessibility of the dyestuff to the cell. The formation of droplets is conditioned by the extent to which it forms colloidal aggregates.

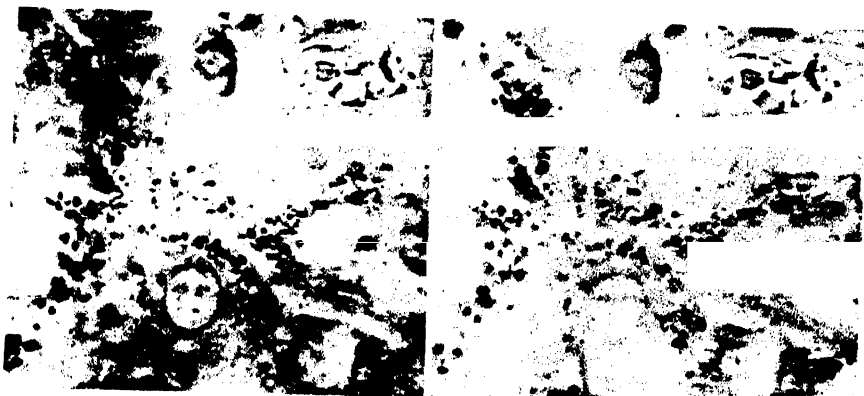
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DESCRIPTION OF PLATE.

Each figure consists of duplicate prints one darker than the other. Murray (1930) has pointed out that these, when viewed through a stereoscope, are effective in bringing out slight differences in contrast. The term "pseudoscopic" has been introduced to describe this method of binocular examination of photomicrographs.

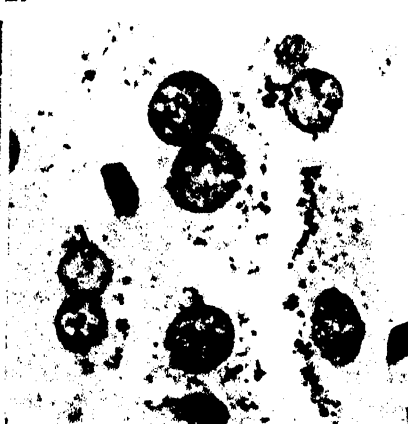
- FIG. 1.—Dye droplets in parenchyma cells of the liver after *intra vitam* staining with trypan blue. Frozen section counterstained with neutral red.
- FIG. 2.—Dye droplets in liver cells after *intra vitam* staining with neutral red. Paraffin section prepared by Gardner's method.
- FIG. 3.—"Krinom" granules or deposits formed in the cells after *intra vitam* staining with neutral red. Paraffin section. Helly fixation. Eosin azure staining.



1.



2.



3.



Recovery Heat in Muscular Contraction without Lactic Acid Formation.

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In a comparison of muscles poisoned with mono-iodo-acetic acid (IAA) in the presence and in the absence of oxygen respectively, Lundsgaard (1930) found :—

- (1) That the spontaneous breakdown of phosphagen in poisoned resting muscle is much more rapid under anaerobic conditions.
- (2) That the onset of the characteristic contracture produced by IAA is accompanied always by an increase in the rate of oxygen consumption.
- (3) That the concentration of IAA which will prevent lactic acid formation, while leaving "respiration" intact, must lie between certain narrow limits ; a small frog's sartorius (*R. temp.*) exposed for 1 hour at 15° C. to a concentration of 1/30,000 in Ringer's solution was sufficiently poisoned to prevent lactic acid production, but might do far more work in oxygen than in nitrogen ; moreover, after a given amount of stimulation, in spite of having done more work, the oxygen muscle might contain far more phosphagen than the nitrogen muscle ;

and having found that the phosphate liberated as the result of phosphagen breakdown may be largely removed by esterification with hexose he suggested :

- (4) That under certain undefined conditions this removal by esterification may be so rapid that oxidative resynthesis of phosphagen may be rendered difficult or impossible.

At Dr. Lundsgaard's suggestion one of us (W.H.) in September, 1930, made a few experiments to find whether the "initial" heat production in an IAA muscle is in any way different from that in a normal one. The result—the complete absence of such a difference—is described by Hartree (1931). Recently

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Fischer (1931, p. 510) has reached the same conclusion, except that he believes the relaxation heat to be somewhat more spread out after poisoning. Following these experiments on the initial heat the delayed heat in oxygen also was studied; it became evident that a normal amount of "recovery" heat might occur after a short tetanus, in a muscle not too heavily poisoned.

The recovery heat can be studied in several ways:—

- (A) By analysing the deflection-time curve after a single short tetanus, by means of a heating-control, as described in a variety of investigations (Hill, 1913; Hartree and Hill, 1922; Hartree and Liljestrand, 1926).
- (B) By comparing the total heat for a series of twitches in oxygen with the total heat in nitrogen (Hill, 1928*b*).
- (C) By stimulating a muscle in nitrogen, measuring the heat, replacing the nitrogen by oxygen and measuring the recovery heat separately.
- (D) By subjecting a muscle to anaerobic conditions at rest and then introducing oxygen.

Each of these methods has been applied to the case of muscles poisoned with IAA, and each has shown the existence of "recovery" heat. Fischer's contrary conclusion (1931, p. 511) is presumably due, as he suggests, to the use of relatively high concentrations (*e.g.*, 1/2,000) of brom-acetic acid, though it may conceivably have been caused by a difference between the bromine and the iodine compounds, or perhaps by some impurity in his brom-acetic acid.

(A) *The Recovery Heat after a Single Short Tetanus.*—The recovery heat production following a short tetanic stimulus in oxygen was investigated first. A pair of sartorii of *R. temp.* (October and November, 100 to 160 mg. the pair) was soaked in Ringer's fluid (p_H 7.4, phosphate 10 mg. P/100 c.c.) and mounted on a thermopile (constantan-iron couples, shellac insulation, vulcanite chamber). A solution of IAA (nearly always 1/25,000) in Ringer's fluid was run into the chamber, and left there for an hour or more with oxygen bubbling through. A longer time of soaking was frequently tried, but if it exceeded 1½ hours there was almost invariably a spontaneous evolution of heat 2 to 2½ hours after the drug was first given. Since ¼ hour had to be allowed, after the solution was removed from the chamber, for a steady zero to be reached, and since each recovery heat record had to be continued for ½ hour in order to ensure that the final zero was reliable (although the recovery heat itself ceases earlier) even the first record was likely to be spoilt by this spontaneous heat production if soaking was too long. Exposure for 1 hour to a 1/25,000 solution probably gave quite sufficient time at room temperature (16° to 17½° C.) for the drug

to have its characteristic effect, since the spontaneous evolution of heat then usually occurred $2\frac{1}{2}$ to 3 hours after the drug was first given and visible contracture at 3 hours. These times refer to the case of a muscle in oxygen after soaking; in nitrogen they were far less—which confirms Lundsgaard's point (1) above.

Note on the Estimation of the Recovery Heat.—Towards the end of the recovery heat records, after 20 minutes or so when recovery was probably complete, the galvanometer always showed a small positive deflection, usually less than 5 mm. out of a maximum deflection of 500 mm. (representing the initial heat). This small deflection was observed for a further 5 or 10 minutes and then it could be seen what allowance was necessary (uniformly distributed over the time of the whole record) on account of zero-motion, etc., to make the final readings after 15 or 20 minutes constant. The amount of this constant was then subtracted from all the readings, from the start of the record, when calculating the total heat. Even if there be some doubt of the correctness of this procedure it does not affect the difference between the recovery heats before and after poisoning, since, fortunately, the mean of eight determinations of the final constant deflection was found to be exactly the same before as after poisoning. If the procedure be omitted altogether all the recovery heats appear to be about 0.3 greater (expressed as a fraction of the initial heat). In the following, only those experiments have been taken into account in which the final divergence from the initial zero was quite small. In many the divergence was larger, owing to the onset of the spontaneous heat production referred to above.

The stimulus was a maximal tetanus directly applied for 0.25 to 0.4 second. In eight apparently very good experiments, in which the muscles were certainly effectively poisoned (as shown by subsequent spontaneous heat production and contracture) the ratio of total recovery heat to initial heat was as follows: 1.2, 1.1, 1.0, 1.0, 1.1, 1.05, 1.05, 1.1. Mean about 1.1. In seven good experiments before poisoning, three of which were on muscles subsequently poisoned and coming in the above series, the recovery heat was also about 1.1.

The earlier contractions therefore of muscles not too heavily poisoned show exactly the same total recovery heat as normal muscles.

On analysing the results, however, a distinct difference appeared in the rate of the recovery heat production. Whereas, before poisoning, the maximum rate was 0.0075 of the initial heat per second, occurring at about 45 seconds after the stimulus (mean of three results), after poisoning it was 0.0049 occurring at about 75 seconds (mean of eight results). Corresponding to the lower rate near the start the rate was higher later on (after 4 minutes). See fig. 1. In several of the earlier experiments, in which the time of soaking in the solution was $1\frac{1}{2}$ hours or more, the total recovery heat could not be even roughly estimated, because of the early spontaneous heat production; it was

obvious, however, from the numbers at the beginning of the record, that the maximum rate of recovery heat production was sometimes much less than that

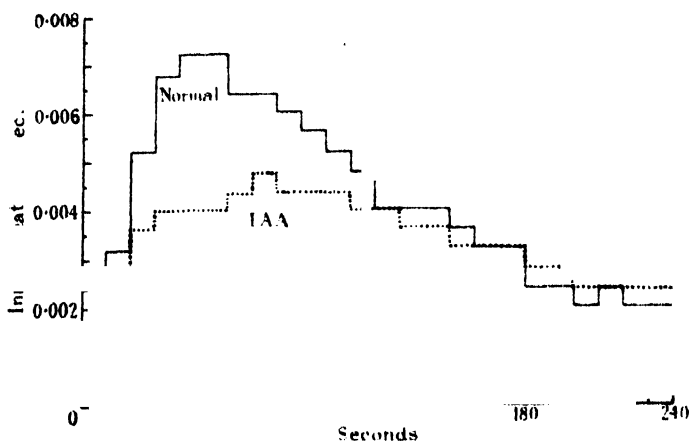


FIG. 1.—Analyses of the first 4 minutes of the recovery heat after a tetanus of 0.3 second. Sartorii of *R. temp.*, November 10, at 17° C. The analyses were made in 10 second intervals, so the stepped lines show the heat occurring during each 10 seconds; for the first step the method gives only an approximate result. *Full line*, muscle unpoisoned in oxygen. *Dotted line*, same muscle after soaking for 1 hour in oxygenated IAA solution, 1/25,000, in the thermopile chamber. The solution was replaced by oxygen and 20 minutes allowed for the galvanometer to settle. The total recovery heat in each case was approximately 1.1 times the initial heat, and took 15 to 20 minutes.

given above. This probably means that, in such cases of longer soaking, the recovery heat is spread out over a still greater time. This slowing of the recovery heat production under the influence of iodo-acetic acid is in agreement with Lundsgaard's conclusion (3) above that the drug interferes with respiration, though in a lesser degree than with lactic acid formation.

(B) *The Recovery Heat for a Series of Twitches.*—A pair of muscles (*R. temp.* or *R. esc.*) was mounted on a thermopile and soaked in Ringer's fluid in a thermostat at 20.3° C. The thermopile had silver-constantan couples, paraffin-shellac-bakelite insulation, and a glass cover. After a sufficient period of soaking the Ringer's fluid was replaced by oxygen. As soon as a steady base line was obtained a series of maximal single shocks was given (about 30 in 2 minutes) and the total heat determined from the area of the deflection-time curve of the galvanometer (see Hill, 1928*b*, p. 185). The twitches were isometric and their tensions were recorded on a smoked drum. The total heat in oxygen divided by the sum of the tensions ($H_0/\Sigma T$) was calculated.

In some experiments the oxygen was then replaced by nitrogen and the

procedure repeated, the quantity $H_N/\Sigma T$ being obtained. Oxygen was then introduced again, and recovery allowed to occur.

Next an IAA-solution in Ringer's fluid was introduced and well stirred by bubbling oxygen. After 45 minutes the solution was replaced by oxygen and when a steady base line was obtained the procedure repeated a third time. The quantity $H_P/\Sigma T$ was obtained, P representing the poisoned muscles.

Details of the experiment and the method of calculating the results are shown in fig. 2. The only difficulty, or uncertainty, arises from the fact that the resting heat rate in oxygen, above which the heat due to stimulation is measured, increases considerably as the result of activity. The method of making the necessary allowance is described in the legend of fig. 2. This increase in the resting heat rate in oxygen is further discussed below.

It was shown by Hill (1928*b*) that, on the average, $H_0/\Sigma T$ is about 2.1 times $H_N/\Sigma T$. In Table I, therefore, the heats are multiplied in each experiment by

Table I.—Recovery Heat of Muscles Poisoned with Mono-iodo-acetic Acid when undergoing a Series of Twitches in Oxygen.

The total heat was measured in each case from the area of the deflection-time curve (see fig. 2). With normal muscles in oxygen the correction necessary for shift of zero between the beginning and the end of a given set of observations was negligible. With IAA muscles there was always a relatively large increase in the resting heat rate in oxygen after stimulation and "recovery," and allowance was made as described in the legend of fig. 2. With normal muscles in nitrogen the correction necessary for vapour pressure change was small and was made in the same way.

The total heat in a series of twitches, when divided by the sum of the tensions developed, $H/\Sigma T$, was given the arbitrary value 2.1 in oxygen [2.1 is the average value of the ratio (heat in O_2) : (heat in N_2), see Hill, 1928*a*].

Date.	IAA.	Oxygen.	Nitrogen.	After IAA.
February 5	1/20000	2.1 (30)	—	1.58 (31)
" 6	1/20000	2.1 (31)	—	1.76 (31)
" 7	1/31000	2.1 (31)	—	1.79 (30)
" 9	1/31000	2.1 (35)	—	1.66 (34)
" 10	1/16700	2.1 (30)	1.13 (30)	1.51 (30)
" 16	1/25000	2.1 (31)	0.96 (29)	1.63 (31)

Notes.

- (1) The duration of soaking in IAA was always 45 minutes.
- (2) In the first experiment tensions were not measured; it is assumed that the tensions in the IAA twitches were the same as in the normal twitches.
- (3) Number of twitches given in brackets: about 2 minutes' stimulation in each case.

a suitable factor, to make $H_0/\Sigma T$ equal to 2.1. In the two experiments in which $H_N/\Sigma T$ was determined it had a value of about unity, as it should. In

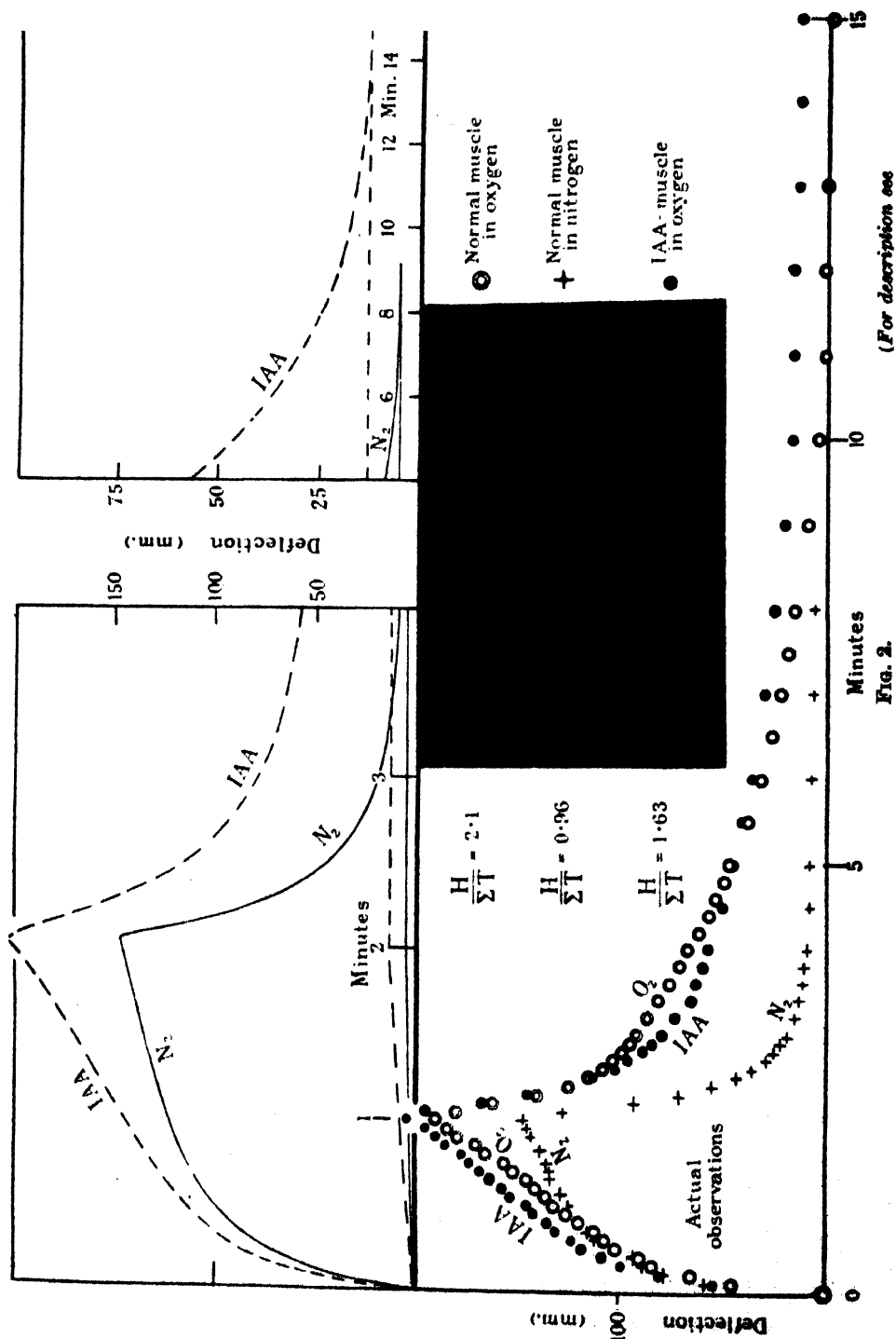


FIG. 2.—Experiment of February 16. *R. esc.* 20.3° C. Lower half of figure, deflection-time curves of galvanometer connected to thermopile during 2 minutes' stimulation. Actual observations. Inset: record of isometric twitches of the three series—(i) top and hollow circles, muscle in oxygen; (ii) middle and crosses, same muscle in nitrogen; (iii) bottom and full circles, same muscle in oxygen after 45 minutes in 1/25,000 iodo-acetic acid. Upper half same experiment, curves drawn for N₂ and IAA from the observations. Different scales in the two parts (right and left) to exhibit the curves better. The total heat is given by the area of a curve above its appropriate "base-line." Each base-line is drawn horizontally from the final position on the right, till it meets the vertical at the end of stimulation. From this point it is drawn straight to the origin. The displacement of the base-line in the nitrogen experiment was due to the increased depression of vapour pressure; in the IAA experiment it was due to the increased resting heat production in oxygen always found after IAA poisoning; in the oxygen experiment there was practically no displacement. Total heat divided by total tension ($H/\Sigma T$) was given the arbitrary value of 2.1 for oxygen. For nitrogen it was then 0.96, for oxygen after IAA 1.63. There is clearly considerable recovery heat in the IAA-muscle.

all the experiments $H_P/\Sigma T$ is far above unity, averaging about 1.65. No great significance is to be attached to the precise value of the average, since the concentration of the poison varied from one experiment to the other. There is no doubt, however, and this is most clearly seen in the upper curves of fig. 2, that a considerable amount of recovery heat occurs. The muscles were all adequately poisoned—they all showed the characteristic contracture fully developed in later stimulation—but no contracture or "spontaneous heat" was to be detected in the course of the observations themselves.

It was shown by Fischer (1931) and by Meyerhof, Lundsgaard and Blaschko (1930) that, in a twitch, the ratio of initial heat to tension developed is unchanged by IAA poisoning. This very important fact we have verified in special experiments described below. Thus, if there were no recovery heat in the IAA-muscle the quantity $H_P/\Sigma T$ should be about unity; the fact that it is about 1.65 means that the recovery heat is about 0.65 of the initial heat, which is about 60 per cent. of its normal amount. It may be that we have over-allowed for the shift of base line of the IAA-muscle; it would be easy and reasonable to allow less; in that case the recovery heat might be made a rather greater fraction of its normal value. The full recovery heat in this type of experiment cannot apparently be found, but a large fraction of it can.

(C) *The Recovery Heat Determined by Admitting Oxygen after Anaerobic Stimulation.*—The recovery heat production can be separated from the initial heat production, and independently measured, by a method described by Hill (1928*b*, p. 188). The muscle, lying on a thermopile in nitrogen, is stimulated by a series of induction shocks and its heat determined. Oxygen is then

introduced, recovery sets in at once, and the heat associated with it is measured from the area of the deflection-time curve of the galvanometer. At 20° C. recovery from 50 twitches is complete in less than half an hour, and the galvanometer returns to, or nearly to, its original position. This method has been applied to muscles poisoned with iodo-acetic acid, with results given in Table II and fig. 3.

Table II.—“Recovery” Heat on Introducing Oxygen to Muscles Poisoned with Iodo-acetic Acid (IAA) and Stimulated in Nitrogen.

Sartorii of *R. esc.*—Recovery heat as a fraction of heat produced by contractions in nitrogen, (A) calculated from curved base line (broken line, fig. 3), (B) calculated from horizontal base line (dotted line, fig. 3). All muscles on stimulation later showed the typical IAA contracture.

Date.	IAA.	Time in N ₂ before O ₂ .	No. of twitches.	Recovery heat.	
				A.	B.
		minutes			
November 27 (1)	1/25000 for 46 minutes	53	52	1.38	0.85*
“ 27 (2)	1/25000 for 45 minutes	77	54	1.59	1.38
“ 28 (1)	1/25000 for 45 minutes	43	52	0.69	0.60
“ 28 (2)	1/25000 for 47 minutes	58	52	0.69	0.47
February 2	1/31250 for 45 minutes	35	49	1.21	1.21
“ 3	1/20000 for 45 minutes	35	30	0.82	0.41*
“ 4	1/31250 for 45 minutes	40	44	0.80	0.65*
“ 5	1/31250 for 60 minutes	45	30	0.74	0.60
“ 6	1/16700 for 45 minutes	30	41	0.35	0.15
“ 9	1/16700 for 45 minutes	36	32	0.71	0.50
“ 10	1/12500 for 45 minutes	—	36	0.35	0.27*

* Fig. 3.

A pair of frog's *sartorii* was mounted on a thermopile and placed in Ringer's fluid (p_H 7.2, 10 mg. P/100 c.c.). After a sufficient time a solution of IAA in similar Ringer's fluid was introduced and stirred by bubbling oxygen. This Ringer's fluid was withdrawn, and replaced by nitrogen. The muscles were stimulated by a series of shocks, one every few seconds, and the heat recorded. When the galvanometer had returned to a steady position oxygen was introduced slowly (so as to cause no disturbance), and the deflection followed for 30 minutes or more, as shown in fig. 3. The area of the deflection-time curve gives the recovery heat.

In the case of muscles poisoned with IAA a difficulty arises in measuring the recovery heat owing to the fact that the galvanometer fails to return to its original position (see fig. 3). It returned in only one (February 2) of the experiments of Table II. It is evident enough that there is a relatively large recovery

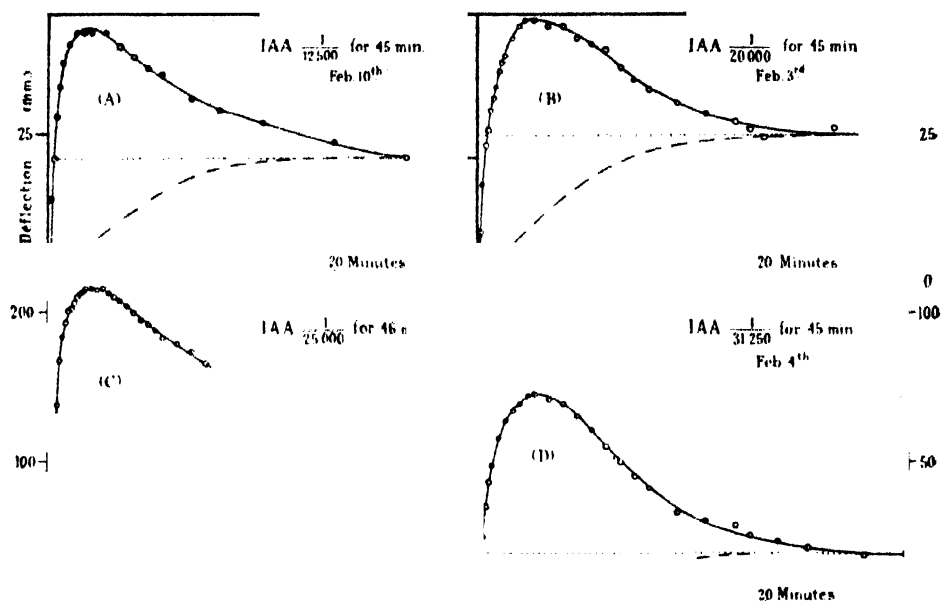


FIG. 3.—“Recovery” heat production in oxygen of muscles poisoned with iodo-acetic acid (IAA) and stimulated in nitrogen. At time zero the galvanometer deflection is represented by 0; oxygen introduced at time zero causes a rapid deflection due to the recovery heat. The final level shown to the right after 30 minutes is joined to the origin by the broken line representing an “assumed” basal heat production. The recovery heat is the area above the broken line. Since this procedure is somewhat arbitrary a minimum value also has been found by calculating the area above the dotted line drawn horizontally to the final level of the curve. The circles represent actual observations of the galvanometer deflection. Note that the curves are not drawn to the same scale of heat production. For details see Table II.

heat production, even in the case of muscles poisoned with as much as $1/12,500$ of IAA. To measure it, however, requires the assumption of some particular “base-line.”

In none of the experiments recorded in Table II did contracture set in until provoked by subsequent stimulation. The large difference of steady heat rate between the muscle initially in nitrogen and finally in oxygen is characteristic of IAA poisoning, and can be found in muscles stimulated some time previously to complete exhaustion. The phenomenon is discussed in detail below. It seems that the base-line, representing the steady resting (as distinguished from the recovery) heat production should be drawn somewhat as shown by the broken lines rising from the origins in fig. 3. The recovery heat is then represented by the area of the curve above this line. It may be, however, that the basal heat production rises more rapidly to its final value,

perhaps as soon as oxygen becomes available by diffusion. In that case the broken lines should have been drawn more to the left and the recovery heats are less. It is certain, however, that the recovery heat cannot be less than the area of the curve above the dotted line drawn horizontally through its final level. In every case, therefore, we have calculated two values, designated (A) and (B) in Table II. Of these, (A) represents the recovery heat above an estimated basal level given by such broken lines as are shown in fig. 3, while (B) represents the minimum recovery heat above the dotted line drawn through the final level of the curve. In Table II these quantities are expressed as fractions of the heat liberated by previous stimulation in nitrogen. In normal unpoisoned muscles the fractions in question would be nearly the same and about equal to 1.1.

There is one small error, of which no account has been taken, which would tend to make the recovery heat ratio, as calculated, slightly too high. In addition to the heat set free anaerobically in the 30 to 54 twitches preceding recovery, the muscles had always been in nitrogen for some time before oxygen was admitted. Their anaerobic heat production during this time should, had we known its amount, have been added to that due to activity. The correction, although not negligible, cannot be great. The large value in the experiment of November 27 (2) may be due in part to the long time (77 minutes) of previous anaerobiosis.

From Table II we see that, whichever method of calculation is adopted, the recovery heat in oxygen is a considerable fraction of the heat in nitrogen which preceded it. The lower values tend to be associated with the higher concentrations of IAA, the higher values with its lower concentrations. We have avoided experiments with concentrations higher than $1/12,500$; even that concentration tends to cause the spontaneous onset of the chemical breakdown and mechanical contracture characteristic of the poison.

(D) *The Heat Production at Rest of Muscles Poisoned with Iodo-acetic Acid.*—Before a muscle poisoned with IAA is stimulated, or—if unstimulated—until sufficient time has elapsed to allow the spontaneous breakdown and onset of contracture to occur, there is nothing to distinguish its rate of heat production at rest, either in oxygen or in nitrogen, from that of a normal muscle. When, however, it has been stimulated, as is seen in figs. 2 and 3 above, there is a relatively large increase in its rate of heat production at rest in oxygen. After recovery from 30 twitches the rate of heat production in oxygen at 20°C . is, on the average, about 80 g. cm. per gramme per minute greater than it was before, roughly a 50 per cent. increase. After stimulation to exhaustion the rate of heat production in oxygen remains very high for long periods, as the following experiments at 20.3°C . on frogs' sartorii show :

Experiment 1. November 20.—IAA 1/25,000 for 45 minutes, then in oxygen. Stimulated to exhaustion and left in oxygen. When a constant deflection had been reached, nitrogen was introduced. The rate of heat production decreased by 370 g. cm. per gramme per minute.

Experiment 2. November 20.—IAA 1/50,000 for 46 minutes, then in oxygen. Procedure as in experiment 1. Rate of heat production decreased on removing oxygen by 372 g. cm. per gramme per minute.

Experiment 3. January 19.—IAA 1/14,000 for 45 minutes, then in nitrogen. Stimulated to exhaustion and final resting heat rate in nitrogen measured. Oxygen then introduced. After a partial recovery process the rate of heat production became constant (at least up to 95 minutes) at 267 g. cm. per gramme per minute in excess of its level in nitrogen.

Experiment 4. January 23.—IAA 1/15,600 for 45 minutes, then in nitrogen. Procedure as in experiment 3. In oxygen the rate of heat production became constant (at least up to 160 minutes) at 533 g. cm. per gramme per minute in excess of its level in nitrogen.

The effect found cannot be due to a change of vapour pressure. In experiments 1 and 2 the removal of oxygen produced the effect noted within 25 minutes, and any slight increase of osmotic pressure due to its removal would work in the opposite direction. In experiments 3 and 4 the introduction of oxygen may have reduced the osmotic pressure somewhat, by a process of partial recovery, but this also would tend to diminish, and not to increase the effect observed.

The high rate of oxidative heat production sometimes lasts for a very long time. More than once the muscle has been left on the thermopile all night, and the deflection in oxygen has been much the same next morning. That this deflection is due to a real heat production—not to a change of vapour pressure—is easily verified by replacing the oxygen by nitrogen, when the deflection becomes considerably less. In various experiments by one of us (W.H.) in which the total heat in nitrogen was measured, whether as the result of stimulation or of the spontaneous breakdown which ultimately occurs, when the deflection was steady oxygen was introduced; after an early deflection of about 130 mm., representing a partial recovery process, the galvanometer finally came back to a level about 70 mm. in excess of its level in nitrogen; this 70 mm. could be abolished by removing the oxygen again. The increased heat production due to oxidation was in these cases (at 17° to 18° C.) about 280 g. cm. per gramme per minute.

The rate of heat production of a normal frog's muscle at rest at 20° C. in oxygen (Hill, 1928a) is of the order of 160 g. cm. per gramme per minute. The removal of oxygen would reduce this to about 65. Thus a muscle which has been stimulated to exhaustion after treatment with IAA has a rate of heat production in oxygen which is considerably greater than that of a normal

muscle. Lundsgaard (1930, p. 66) found that the rate of oxygen consumption of an IAA muscle increases very much when contracture sets in. In the normal muscle it is supposed that the processes which go on in nitrogen are reversed and completed in the presence of oxygen; in the absence of oxygen an "oxygen debt" is incurred which is "paid off" later when oxygen is admitted. It is interesting to inquire whether the same occurs in an exhausted IAA muscle.

When an IAA muscle is stimulated to complete exhaustion and left in oxygen its rate of heat production becomes steady at a high level. When the oxygen is removed the rate of heat production falls as shown in fig. 4, with a certain

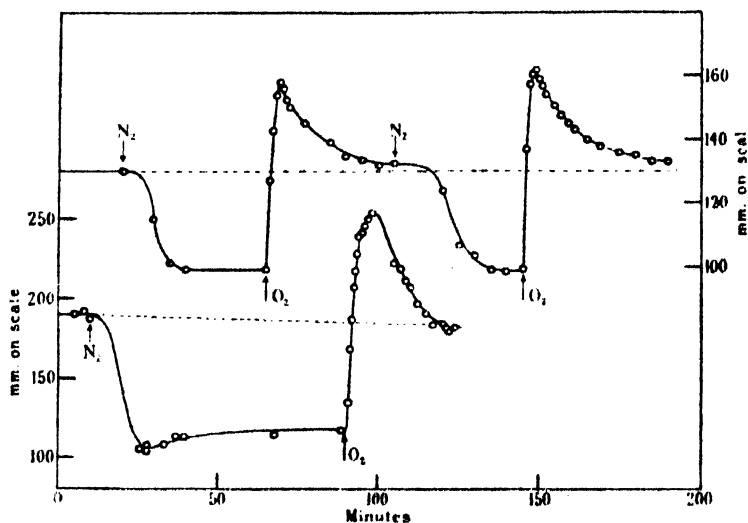


FIG. 4.—To show "oxygen debt" of IAA muscles. 20.3° C. Circles represent actual observations on galvanometer scale. *Upper curve*, experiment of February 6, *R. esc.*, sartorii, IAA 1/16,700 for 45 minutes, muscles exhausted in oxygen 110 minutes before first observation shown. N_2 replaced O_2 , and O_2 replaced N_2 , at arrows as indicated. *Lower curve*, experiment of February 3, *R. esc.*, sartorii, IAA 1/20,000 for 45 minutes, muscles in oxygen 60 minutes before first observation shown.

lag corresponding to the time required for oxygen already present to be used up, or to diffuse away. When oxygen is readmitted the rate of heat production instantly rises and reaches a value well above the final steady level, to which it falls again in 30 to 50 minutes. It is clear that an "oxygen debt" has been incurred, in the sense that in oxygen, after anaerobic conditions, an excess of heat is given out corresponding to part of the deficit of heat during the period of oxygen want. It is clear also that the heat excess in oxygen is considerably less than the heat deficit in nitrogen. Special experiments have been made to

verify this, a comparison being made of the heat excess after a short and after a long period of oxygen want.

A "recovery" process of some kind, therefore, can go on in a muscle poisoned with IAA, even after complete exhaustion. In the absence of oxygen for a time some sort of breakdown continues to occur which requires oxygen to complete it; its products accumulate and when oxygen is re-admitted a reaction takes place between the oxygen and these accumulated products. It is clear, however, from the fact that the heat excess *after* is so much less than the heat deficit *during* oxygen want, that a large part of the oxidation occurring in the exhausted muscle is not a consequence of these preceding reactions; the heat in oxygen must be due to the oxidation of bodies which, if oxygen be absent, do not accumulate but merely are not oxidised.

A curious and suggestive parallel exists between the observations recorded here on muscles poisoned with IAA and those in a previous paper (Hill, 1929) on muscles kept for long periods in oxygen-free Ringer's fluid. Muscles which were in "good" condition after survival showed a normal heat-rate in oxygen; muscles which were in "poor" condition showed an abnormally high value. In the "good" muscles the heat rate in oxygen was, on the average, about twice that in nitrogen; in the "poor" muscles it was more than three times as great. In the "good" muscles the recovery process after oxygen want followed a normal course, the galvanometer returned to somewhere near its initial position; in the "poor" muscles, after oxygen want a large deflection occurred and persisted apparently indefinitely; fig. 2 of the paper referred to is remarkably similar to an extreme case of the phenomenon shown in fig. 3 of the present paper for muscles poisoned with IAA. Apparently, as was stated in the 1929 paper, "in the dead or dying muscle at rest oxidations can proceed at a greater rate than in the fresh surviving tissue. It may be that if oxygen want is pressed to a degree at which partial, or complete, failure of the muscle sets in, disintegrative processes are released, in which enzymes or substances, normally controlled in the organised life of the cell, react at random with oxygen and liberate an excess of heat." The interference with the mechanism provided by poisoning with IAA seems to release the same oxidative processes as prolonged anaerobic survival. In both cases some power of "recovery" from anaerobic conditions appears to remain, as shown by the heat excess in oxygen following a heat deficit in its absence. This, however, may mean nothing more than that a fraction of the oxidation is concerned with the completion of reactions which normally run part of their course in the absence of oxygen.

The Absence of an Effect of Iodo-acetic Acid on the Isometric Heat Coefficient.

—The experiments on the equality of T/H , in a shock or a short tetanus, before and after IAA poisoning, were performed as follows. The muscles in every case were in oxygenated Ringer's fluid at 20.3°C ., on a thermopile and attached to a tension lever. The values of T and H were determined with a series of maximal direct stimuli at equal intervals, until a constant value of T/H , or a slowly and steadily changing value, was obtained. The appropriate amount (1 to 2 c.c.) of 0.4 per cent. IAA solution was then introduced and mixed with the Ringer's fluid, and the series continued until contracture set in. The results are given in fig. 5. It is not possible to obtain extremely regular values of the heat production of a muscle in Ringer's fluid. In a gas the muscle sticks to the thermopile under the surface tension of liquid adhering to it; in a solution it tends more to float in the fluid, and slight irregularities of contact give random variations in the heat deflection observed; the tensions are more uniform. A mean line, however, drawn through the plotted observations of T/H gives a reliable result, and it is seen from fig. 5 that no regular change occurred, as the result of introducing IAA, even in some cases when contracture was obviously beginning to set in. The muscles were certainly effectively poisoned, as shown by the contracture which ultimately occurred in every case, and experience has shown that such concentrations as 1/10,000 of IAA cause complete poisoning within half an hour. There is no doubt, therefore, as shown by Fischer (1931) and by Meyerhof, Lundsgaard and Blaschko (1930), that T/H in a twitch, or in a short tetanus, is entirely unaffected by preventing the formation of lactic acid in contraction.

It has no immediate bearing on the subject of the present paper but it is of interest to record that the effects of work, and of length, on the heat production occur unchanged in muscles poisoned with IAA. The Fenn effect and its modification described by Rothschild, together with all the relations between these and the length of the muscle, were investigated by the methods described by Hill (1930) and found to be the same as in the normal muscle. These adjustments, therefore, adopting Lundsgaard's view of the mechanism (1930, p. 81) occur in the primary change by which phosphagen is broken down, and are not a property

FIG. 5.—Seven experiments at 20.3°C . showing that in a single twitch, or a short tetanus, the ratio of tension developed to heat produced (T/H) is the same before and after treatment of the muscle with iodo-acetic acid: muscles in oxygenated Ringer's fluid on a thermopile, stimulated at regular intervals. At the first arrow IAA-solution, to give the concentration noted, was introduced into the chamber. Observed values of T/H (arbitrary units) shown before IAA by full circles, after by hollow circles. Note that no regular change occurred in the value of T/H , as the result of introducing IAA, even in some cases where contracture had obviously begun to develop.

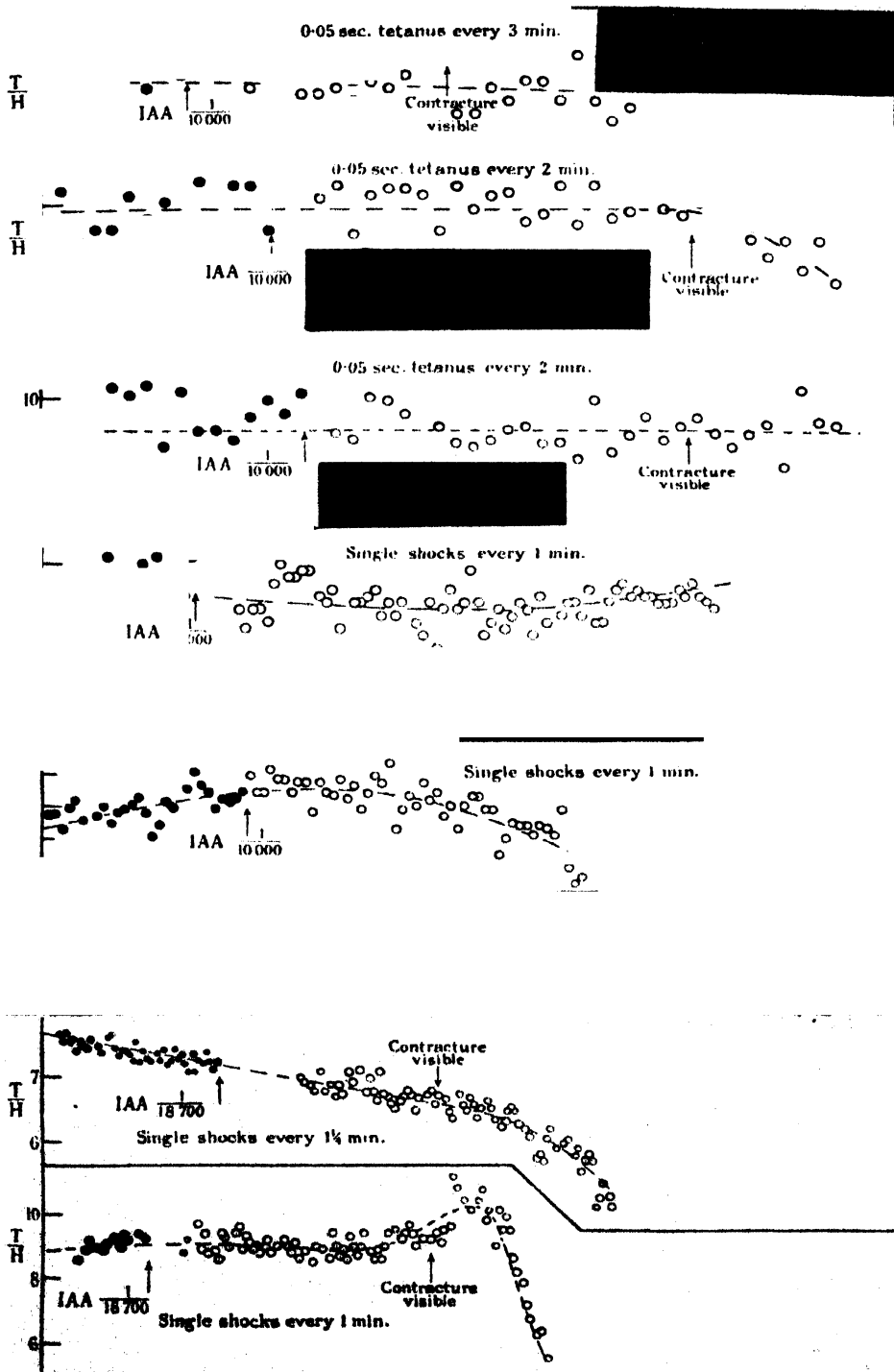


FIG. 5.

(For description see previous page.)

merely of the restoration processes in which the energy of lactic acid formation is employed to effect phosphagen resynthesis.

The Recovery Heat of Normal Muscles after Complete Exhaustion.—In a muscle poisoned with IAA, (i) the recovery heat observed in oxygen after stimulation in nitrogen is usually (Table II) less than the anaerobic heat which preceded it, and (ii) the recovery heat in a series of twitches in oxygen is less than in a similar series by a normal muscle (Table I). In spite of the high capacity for oxidation exhibited by an IAA muscle the power of physiological recovery is largely abolished and the excess energy liberated after activity is diminished. This is possibly due in part to the "side-tracking" of chemical bodies normally calling for restoration in recovery; for example, in an unpoisoned muscle moderately stimulated the creatine and phosphate liberated by the breakdown of creatine-phosphoric acid are resynthesised in recovery; in the IAA muscle, however, they cannot be, since the phosphate has been esterified with hexose and so removed (Lundsgaard, 1930). A curious and possibly significant parallel to the case of the IAA muscle exists in that of an unpoisoned muscle stimulated to complete exhaustion (see Hill, 1928*b*, footnote, p. 189). In this, "recovery is not complete, or is extremely protracted." The recovery experiment shown in fig. 6 illustrates the phenomenon.

This curious effect has been known to us for some years and has been noticed many times, but no explanation of it was forthcoming. Its similarity to that which occurs in the IAA muscle encouraged us to make two more experiments to verify its existence. The actual observations in one of these are shown in fig. 6: in both the recovery heat was about 25 per cent. of the heat in a preceding anaerobic stimulation to exhaustion, instead of the 100 per cent. found after moderate stimulation (see Hill, 1928*b*, p. 189).

In the recovery observations the deflection becomes quite constant after about 100 minutes, usually slightly above its level before oxygen was let in. At the end of the stimulation curve there is a definite increment in deflection (in fig. 6 about 80 mm.) due to change of vapour pressure (Hill and Kupalov, 1930). In both cases, therefore, the area must be measured above an appropriate base-line: this introduces a small arbitrary factor into the calculation, of the kind discussed in connection with figs. 2 and 3; its amount, however, is so small that it cannot obscure the main effect.

When the muscle has "recovered" as shown in fig. 6, it is usually inexcitable, and even prolonged washing with oxygenated Ringer's fluid, although it will bring back excitability (Dulière and Horton, 1929; Horton, 1930), will not restore it to good condition. Clearly something has gone amiss. According

to Eggleton and Eggleton (1929, p. 204) rapidly induced fatigue causes a disappearance of phosphagen and an increase of orthophosphate. The two

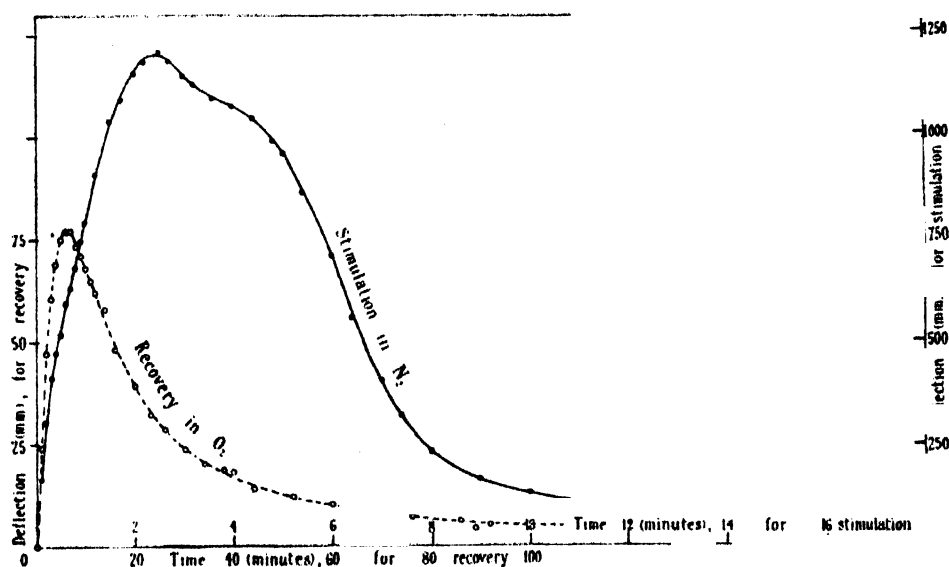


FIG. 6.—To show recovery heat in oxygen, after stimulation to extreme exhaustion of a normal muscle in nitrogen; for comparison, the heat during stimulation also is shown. Vertically, galvanometer deflection: horizontally, time. Heat given by area of deflection-time curve. Note that the curves are drawn for stimulation and for recovery with the same scale of area, i.e., of heat production, but with very different scales of time and deflection. Circles represent actual observations. Sartorii of *R. esc.*, January 16, 20.3° C. on thermopile. After appropriate soaking in Ringer's fluid, p_H 7.2, 10 mg. P/100 c.c., stimulation was applied in nitrogen, 45 maximal break shocks per minute for 18 minutes, till the muscles were completely exhausted. Deflection-time curve (full circles) observed for total heat on stimulation. The final steady deflection was caused by condensation due to fall of vapour pressure. When the galvanometer was perfectly steady again oxygen was slowly introduced and the deflection-time curve was observed for 92 minutes. Its area gives the recovery heat. Note that both curves start from the origin and that the recovery heat in oxygen is only about one-quarter of the preceding heat by stimulation in nitrogen.

changes, however, are not equivalent, 20 to 40 per cent. of the phosphagen phosphorus reappearing as a hexose phosphoric ester. In the extreme fatigue referred to here it may be that, as in an IAA muscle, a still greater fraction of the phosphate freed from phosphagen is esterified: if so, it is "side-tracked" and cannot, presumably, be restored in recovery, which may be one reason why recovery is incomplete. The matter deserves further chemical investigation.

The Physiological Recovery of Muscles Poisoned with Iodo-acetic Acid.—

Lundsgaard (1930, fig. 3, p. 69) showed that it is possible sometimes to adjust the conditions (concentration of IAA, etc.) so that a poisoned muscle gives a greater number of twitches in oxygen than in nitrogen; this means that physiological recovery—as distinguished from “recovery” heat production—must in these cases have occurred. He admits, however, that it is not easy to find the right conditions. The results obtained in May could not always be repeated in July. It is probably a question of the relative effects of IAA on two enzyme systems; the one concerned with the lactic acid forming mechanism, the other perhaps with that by which, with energy supplied by oxidation, phosphagen is restored. It is apparently not easy to interfere with the former by IAA without equally affecting the latter. The “recovery” heat which, as we have shown above, always seems—in some measure—to occur may not, except under special conditions, succeed in effecting functional recovery.

We wished to confirm Lundsgaard's statement and made a number of experiments on symmetrical sartorii poisoned with IAA, one in oxygen (or oxygenated Ringer's fluid), the other in nitrogen (or Ringer's fluid through which nitrogen bubbled). The two muscles were in similar chambers, attached to similar isometric levers, and stimulated about 6 times per minute by just maximal break shocks at the same frequency. Nineteen experiments were performed on the muscles of Dutch *R. esc.* Of these only one showed any superiority in oxygen, the rest no significant difference. The range of concentrations explored was from 1/12,500 to 1/50,000.

It was then noticed that Lundsgaard had used muscles from *R. temp.*, and eight experiments were performed on these, of which six gave a greater total tension in oxygen than in nitrogen. In two experiments there was no difference. The positive effect of oxygen occurred in either sex. The results, roughly estimated, were as shown in the following table. All experiments were at room temperature (about 17° C.), and the soaking in every case was in oxygenated Ringer's fluid containing the poison. The muscles were small, usually about 50 mg. each. Only after exposure to the poison were the conditions, as regards oxygen, made different in the two muscles.

	Date.			
	January 29.	January 30.	January 30.	January 30.
IAA for	1/25000	1/30000	1/30000	1/30000
Minutes	52	60	40	80
ET (oxygen)/ET (nitrogen)	2½	2½	1	1

Date.

	January 30.	January 31.	January 31.	February 1
IAA for	1/30000	1/30000	1/30000	1/16700
Minutes	58	60	60	45
ET (oxygen)/ET (nitrogen)	2	1½	2½	2

The last two experiments are shown in fig. 7. It is clear that functional recovery can occur, but that it often may not, and it is curious that the two

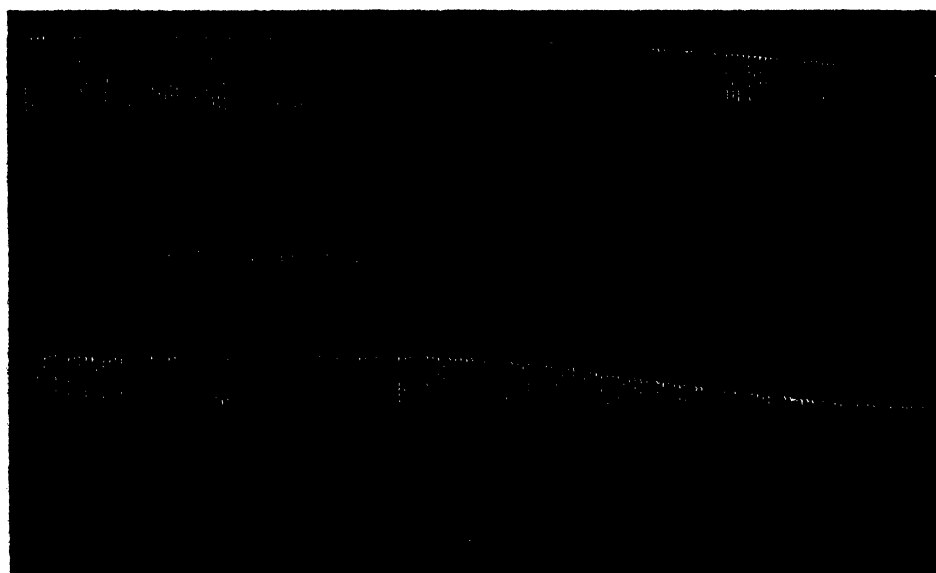


FIG. 7.—Functional recovery in oxygen of stimulated IAA muscles. Symmetrical sartorii of *R. temp.* at room temperature. Muscles mounted in two similar chambers and connected to two similar isometric levers; poisoned by soaking in oxygenated Ringer's fluid containing IAA, then one in oxygen, the other in nitrogen, and stimulated about 6 times per minute by just maximal break induction shocks at the same frequency, recording one above the other (O, upper record) on the same drum. Tension downwards, read from left to right. Upper pair after IAA 1/30,000 for 60 minutes. Lower pair after IAA 1/16,700 for 45 minutes.

species (*R. temp.* and *R. esc.*) should differ so much (at least in January) in this respect.

The Spontaneous Heat Production and Contracture.—In two experiments in which the muscles were not stimulated, after being put in the IAA solution

(1/25,000 for 1 hour), the spontaneous heat production in oxygen was observed to rise to its maximum rate, about 500 g. cm. (0.012 cal.) per gramme per minute, before there was any sign of contracture. On the other hand, when the muscles were stimulated after poisoning, contracture was visible very soon after the start of the spontaneous heat, in all but one out of eight experiments. This was the case also in one good experiment in nitrogen in which there was no stimulus. The spontaneous heat has a perfectly definite start, and certainly does not go on all the time after the solution is removed. When no stimulus has been given (or at a sufficiently long time after a stimulus) the galvanometer will remain quite steady for some time and then move off at a quickly increasing rate. Since the scale can be read with certainty to $\frac{1}{2}$ mm., it can definitely be said, within a few minutes, when the spontaneous heat starts.

The total heat in oxygen caused by this spontaneous breakdown is rather large, but it is not easy to determine it exactly owing to the shift of zero which occurs, due to the change of vapour pressure and of resting heat rate. Drawing in an imaginary base-line similar to those of fig. 3 the total spontaneous heat in oxygen (*R. temp.*) is about $1\frac{1}{2}$ cal. per 1 g. and may take 4 hours to appear. Almost the same total heat in oxygen is obtained in about 2 hours by stimulation (*R. temp.*, 0.2 second tetanus every 2 minutes). The total heat in nitrogen due to stimulation (Hill and Parkinson, 1931) is about 0.37 cal. per 1 g. In the stimulation experiments in oxygen the total initial heat was about 0.5 cal. per 1 g. It is clear that a certain amount of functional recovery can occur in oxygen, though most of the "recovery" heat is apparently wastefully expended without producing any physiological restoration.

The Anaerobic Delayed Heat.—Several attempts were made in IAA-muscles to measure the anaerobic delayed heat (if any) after a short tetanus. In the experiments at Cambridge, after 1 hour's soaking in 1/25,000 IAA solution, spontaneous heat production occurred in nitrogen too early to allow a reliable end to the heat record, even after the first stimulus. In a few experiments at University College, after 1/16,700 IAA solution for $\frac{1}{2}$ hour, there was very clear evidence of delayed heat in nitrogen: this also, however, may have been due to spontaneous breakdown following activity, and may not be a part, properly speaking, of the preceding contraction. The matter must be further investigated; if the anaerobic delayed heat in a normal muscle is due to delayed lactic acid formation (Meyerhof, 1931; Lehnartz, 1931) it should be absent in the poisoned muscle in which no lactic acid formation at all occurs. It may be possible to avoid the trouble of the spontaneous breakdown referred to.

Discussion.

Functional recovery from activity, associated with oxidation, can clearly occur under certain circumstances in muscles sufficiently poisoned with iodo-acetic acid to forbid lactic acid formation. Presumably phosphate and creatine are resynthesised to phosphagen, with the aid of energy liberated by oxidation. Clearly also, on the other hand, heat which in a normal muscle would logically be called "recovery heat" can be liberated, without any corresponding degree of functional restoration. The process of activity, or its chemical products, seems to set in train a series of reactions involving oxidation, whether or not these can result in the physiological recovery of the muscle. The high rate of oxidative heat production possible in an IAA muscle stimulated to exhaustion, even after a heavy dose of the poison, shows that there has been no interference with oxidative enzymes as such. *The interference is presumably with the mechanism through which the energy liberated by oxidation is utilised for resynthesis.*

Apparently therefore there are at least two separate effects of iodo-acetic acid, (a) on the system by which lactic acid is formed, and (b) on the system by which the energy liberated by oxidation is used to drive the endothermic processes of recovery. These effects can be separated to a certain extent, since, as we have seen, some degree of functional restoration after activity is possible in oxygen when the lactic acid mechanism has already been eliminated. The coupling of oxidation with recovery has been broken by the second effect. Whether iodo-acetic acid interferes with still other parts of the mechanism is not certain, but the onset of contracture, which is so characteristic of the poisoning, has no obvious connection with either of the other effects.

The rate of heat production in oxygen of a poisoned muscle after exhaustion is very high, say 400 g. cm. per gramme per minute, nearly 14 cal. per gramme per day. If due to the oxidation of carbohydrate this would lead to the disappearance of about 3.5 mg. per 1 g., i.e., 0.35 per cent. The chemical determination of such a loss should be easy. The experiment would have to be performed on thin muscles, to ensure an adequate supply of oxygen. A measurement of the oxygen consumption and of the respiratory quotient would give further information.

Summary.

(1) Muscles poisoned with mono-iodo-acetic acid (Lundsgaard) contract without producing any lactic acid. After contraction in oxygen a delayed

heat production occurs, similar to that which in normal muscles is associated with recovery.

(2) After the first one or two contractions of a poisoned muscle the "recovery" heat may be of normal amount in relation to the initial heat. After 30 or 40 twitches it is less than the normal, but still a considerable fraction of it.

(3) The heat rate in oxygen of a muscle poisoned with iodo-acetic acid and exhausted may remain, for long periods, far higher than that of a normal resting muscle. If the oxygen be removed for a period the heat rate falls, and a "heat-deficit" occurs which is partly, but only partly, balanced by a "heat-excess" when oxygen is readmitted.

(4) In a twitch or a short tetanus, as found by others, the ratio of tension developed to heat produced is entirely unaffected by poisoning with iodo-acetic acid.

(5) Functional recovery in oxygen, after stimulation, can be demonstrated under certain conditions, in muscles poisoned with iodo-acetic acid. As found by Lundsgaard, however, the conditions for such functional recovery are not always easy to obtain. In the muscles of *R. esc.*, for example, recovery could not be demonstrated (January), although in those of *R. temp.* it could.

(6) The persistence of "recovery" heat when functional recovery has been eliminated suggests that one effect of iodo-acetic acid is to interfere with the mechanism by which the energy released in oxidation can be employed in driving the endothermic reactions necessary for functional recovery. It does not interfere with oxidation as such, at least in the concentrations used by us.

(7) In normal muscles stimulated to extreme exhaustion the "recovery" heat has only about one-quarter of its usual value, in relation to the initial heat. It is possible that in normal muscles pushed to extreme exhaustion, as in muscles poisoned with iodo-acetic acid, one reason for incomplete recovery is that phosphate set free by the breakdown of creatine-phosphoric acid is "side-tracked" as a hexose-phosphoric ester, and so cannot be recombined with creatine.

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On the Return of Sensitiveness in Corneal Grafts in Rabbits.

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Introduction.

The object of the present paper is to examine the factors which determine whether or not a piece of cornea grafted from one rabbit on the cornea of another becomes sensitive or remains insensitive—in other words, the factors under which a piece of foreign cornea becomes provided with an afferent nerve supply. A long series of experiments on corneal grafting in rabbits, started in May, 1922, and continued without intermission, has provided ample material. Systematic observations on the sensitiveness of these grafts have been carried out since 1925.

Previous Observations.

Elschnig (1930) in a description of his corneal grafts, a proportion of which were clear, says "it is noteworthy that even in the oldest cases of transparent implant observed, complete sensibility has not developed, but as a rule there does develop a sensibility to heavy touch."

Present Observations.

The present observations are based upon the records of 29 successful (but not necessarily clear) grafts, in 21 of which the graft became sensitive, and in the remaining eight of which the graft did not become sensitive.

Sensitiveness was tested in the following manner. A small twisted tuft of cotton wool was gently stroked across the graft, care being taken that it did not touch the hairs about the eyelids, nor the adjoining cornea; the animal was closely watched for any movement of the eyelids or nictitating membrane—the proper response; various parts of the surface of the graft were tested in this way, and the method of testing was then tried on the surrounding cornea to make sure of the adequacy of the stimulus; care was taken that the pressure exerted on the graft should be as light as possible, in order to eliminate responses to pressure as distinguished from light touch.

In these 29 experiments the operative procedure was varied, and the end results of the operations showed considerable differences with regard to the clarity or otherwise of the grafts, and in other directions.

The object of the present paper is to analyse these differences in relation to the return of sensitiveness.

The transplantation was carried out in the following manner. A portion of the cornea of a rabbit (the host) having been removed under full anaesthesia, and with aseptic precautions, a portion, of approximately the same shape and size, was removed from the cornea of another rabbit (the donor) and placed over the hole in the cornea of the host. Here it was retained by stitches or other method of fixation until physiological union of the graft took place. Various methods of fixation were used—marginal stitches, cross stitches or transverse stitches, were all utilised, and stitching was sometimes not used at all. In some cases the graft was cut with a sloping or shelving margin, and in other cases the cut margin was at right-angles to the corneal surface. None of these technical variations was found to have a direct bearing on the problem of this paper; but the site of transplantation was observed to have such a bearing.

In 23 of the experiments under review, the graft was made more or less centrally in the host's cornea, *i.e.*, over the pupil. In the remaining six experiments the graft was made peripherally, extending to the margin of the cornea, *i.e.*, on a part of the host's cornea which covers the iris.

In a few cases the graft was made simply by removing a part of the cornea of a rabbit and immediately replacing it in the site from which it had been removed. In all other cases the graft was taken from another rabbit. This difference of procedure had no bearing on the results.

(A) Of the 23 central grafts, 16 became sensitive, and seven remained insensitive.

Of these 16 sensitive grafts, in 14 the iris had become adherent to the graft,

while in the cases of the remaining two experiments the iris had remained free ; it therefore follows that an adherent iris is not invariably present in sensitive grafts. Of these 16 sensitive grafts, 15 had united primarily, while one exhibited for a short time a small gap at one side. In their final states, 13 of the grafts were opaque, one was nebulous, and two showed central clear areas (but were opaque round the periphery). Each of the 16 sensitive grafts was found to have become vascularised ; in the case of three, the blood vessels had grown from the adherent iris alone, and in the case of two, from the conjunctiva, but not from the iris.

Of the seven grafts that remained *insensitive*, four had a minute adhesion of the iris—which became completely freed in 19 days (in two cases); 6½ weeks (in one case); and 3 months (in the fourth case). All of these seven grafts had united well. In their final states, one was opaque, one had a central clear area, and the remaining five were clear. In one of these seven grafts, there were present a few blood vessels, and this graft was opaque. Two of the grafts were very slightly vascularised at one point on the margin of the graft, in a very small and localised area, corresponding to the point of adhesion of the iris. The other four were not vascularised at all.

(B) Of the six marginal grafts, five became sensitive, and one did not. The five sensitive grafts each had an adherent iris ; three were of medium size, and two were larger ; one united primarily all round, while four had at first a gap at one side ; all were opaque, and all were supplied with blood vessels. The graft that remained insensitive was opaque and had an adherent iris.

(C) Of the 16 central grafts that became sensitive, eight were sensitive over the whole surface at or before 4 months after the operation, and one of these was sensitive as early as 2 months thereafter. On the other hand, of the five marginal grafts that became sensitive, none did so as early as 4 months—the earliest being 5 months after operation. The average time taken for sensitiveness to return was distinctly less in the case of the central grafts than in that of the marginal grafts. It was found that the peripheral part of a graft became sensitive sooner than its central part, and the earliest return of sensitivity noted at the margins was 6 weeks after operation (in a central graft).

(D) Of the total of 29 grafts, 21 became sensitive and eight remained insensitive. Not one of the sensitive grafts was clear and the opacity in most cases was pronounced ; each sensitive graft was found to have become vascularised.

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The eight insensitive grafts, with one exception, were either completely, or practically, devoid of blood vessels. Of the 23 opaque grafts, 21 became sensitive, while all the five clear grafts remained insensitive. The average time taken for sensitiveness to develop in vascularised central grafts was 5 months, and the average period of observation of the clear insensitive grafts was about 13 months.

(E) The following individual instances are of interest :—

- (i) One central graft became partly detached at its lower border, but united later ; the iris was adherent to the lower margin ; the lower two-thirds of the graft became opaque, while the upper one-third was clear. Sensitiveness returned first to the lower opaque portion, and later to the upper clear portion. This seems to indicate that the vascularised lower portion developed an afferent nerve supply sooner than the avascular upper portion.
- (ii) One graft had an adhesion of the iris, and was vascularised from the adherent iris only. The adhesion of the iris, however, became freed in less than 2 months, and yet the graft became sensitive.

This observation shows that the afferent nerve supply did not solely accompany the blood vessels which grew in from the iris, because any such afferent paths would have been destroyed when the adhesion of the iris gave way.

- (iii) One graft which became sensitive had no adhesion of the iris, but was supplied with blood vessels from the conjunctiva.

Conclusions.

From the above observations it will be seen that the only factors which have a definite bearing on the development of sensitiveness in corneal grafts are those which in effect imply the presence or absence of vascularity.

The perfect corneal graft is one which is essentially clear, and avascular.

All the sensitive grafts were vascularised grafts. All the clear grafts were either completely, or almost completely, avascular, and remained insensitive.

From the results of these experiments the conclusion may be drawn that the establishment of an afferent nerve supply to a corneal graft depends upon a precedent or concurrent growth of blood vessels in that graft. It seems to matter little whether the blood vessels are supplied from an adherent iris, or whether they grow in from the margin of the cornea.

Summary.

(1) In 29 cases of completely united corneal grafts in rabbits, five remained clear and insensitive, one had a central clear area and remained insensitive, and 21 out of 23 opaque grafts became vascularised and sensitive.

(2) Analysis of these experiments seems to show that there is a clear correlation between the vascularisation of a corneal graft and the invasion of it by the afferent nerve fibres upon which depends the return of sensitivity.

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Experiments on Growth and Inhibition. Part II.—New Phenomena of Inhibition.

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Introduction.

If young seedlings of *Pisum sativum* or *Vicia faba* are decapitated in the epicotyl, the axillary buds of the cotyledons grow out, so that plants are formed with two shoots springing from the same level. If one grows a batch of such plants—"two-shoot plants," as they will be called—one finds that in some of them the shoots are very unequal from the start. In these plants the weaker shoot very soon stops growing, often when it is only a few millimetres long, and after some weeks it dies. This is clearly because it is inhibited by the stronger shoot, for if the stronger shoot is removed before the weaker shoot has stopped growing, then the latter goes on growing indefinitely. In other plants in which the shoots are less unequal, the weaker one grows for rather longer before it is stopped by inhibition, and in others again, in which they are very nearly or quite equal, they both grow on indefinitely, or at least for a long time—six weeks or more. (It is stated, indeed, by Mogk (1913) that two such equal shoots sometimes check and finally kill one another. This has not happened in any of the writer's plants, perhaps because they have not been left for a long

enough time. At all events, when the shoots are about equal, it is very much longer before the growth of either is inhibited, if it ever is.)

These facts are well known, but none the less they suggest the interesting question how it is that two equal shoots do not inhibit one another, or at least not completely. It occurred to the writer that if one were to remove from one of them the rapidly developing leaves near the apex, which have been shown to be the inhibiting members (Snow, 1929), the balance between them would be upset, and that the results might throw light on the question. Accordingly the following experiment was arranged.

Experiments.

Experiment 1.—Pea seedlings, of race Thomas Laxton, were decapitated when young, so that two shoots grew out from the axils of the cotyledons. Six plants were selected in which the shoots were equal, or nearly so, and, when they had each expanded two leaves, one shoot of each plant (the slightly longer one, if they were unequal) was defoliated from below upwards until only the very small leaves near the apex remained. The other shoot was left intact. The largest leaves remaining on the defoliated shoots were, in five of the plants, the fifth leaves from the base, and they were between 0.75 and 1 mm. long at the time of operation. In the sixth plant (No. 2 of Table I), the largest remaining leaf was the fourth, and was 1.3 mm. long.

Actually, in this and other similar experiments, the operations were often carried out in two stages, the smallest leaf to be removed being left until a few days later than the others; but this complication is of no importance. Also the lengths of the largest remaining leaves were not directly measured, but were estimated as equal to one-third of the length of the next larger leaf, which was removed (see Snow, 1929, p. 347).

Four other similar cotyledonary axillary shoots, belonging to other plants of the same batch, were arranged as controls at the same time. They were similarly defoliated, but were left each as the only shoot on the plant, the other shoot being removed. The largest remaining leaves on these control shoots were the fifth, and were between 0.8 and 1.1 mm. at the start.

After 13 days from the start of the operations, all the shoots were measured, and their growth was reckoned by subtracting their length at the start. The largest remaining leaves of the defoliated shoots were also measured, and the corresponding leaves of the intact shoots. The results are given in the following tables :—

Table I. Experiment 1. *Pisum sativum*.—Growth in millimetres of Shoots in 13 days.

"Two-shoot plants."		Single-shoot controls.
Six defoliated shoots.	Six intact shoots.	Four defoliated shoots.
4	74	81
22	74	85
9	78	68
4	78	55
11	71	—
35	55	—

Table II. Experiment 1.—Growth in millimetres of largest remaining Leaves of Defoliated Shoots and of corresponding Leaves of Intact Shoots in 13 days.

"Two-shoot plants."		Single-shoot controls.
Six defoliated shoots.	Six intact shoots.	Four defoliated shoots.
0.75	7.0	7.5
1.50	18.0	13.5
1.25	13.0	12.0
1.25	6.0	5.25
1.50	12.5	—
2.00	4.75	—

From these tables it can be seen that in the first five of the "two-shoot plants" the defoliated shoots grew in length very much less than the intact shoots during the first 13 days. Their remaining leaves also grew very much less than the corresponding leaves of the intact shoots. Only in the sixth plant was the difference for some reason less extreme. On the other hand, the similarly defoliated single-control shoots grew meanwhile, and developed their leaves at about the same rate as the intact shoots. By the end of the first 13 days, or possibly sooner, the defoliated shoots of the first five "two-shoot plants" were completely arrested in growth. After this time they gradually turned yellow and limp from the apex downwards, although in several of the plants the cotyledons were not yet exhausted. At 26 days after the operations

they had died nearly down to the base, whereas the intact shoots of the same plants and the defoliated single shoots were healthy and growing.

From these results it is clear that a defoliation of the kind described does not arrest the growth of a pea shoot if it is the only shoot on the plant, nor even retard it more than slightly, if at all. It is only when another intact growing shoot is present on the same plant that a defoliated shoot is strongly retarded, and finally arrested and killed. It therefore appears that when two intact and equal cotyledonary axillaries are growing on one plant, they must be in some sense in equilibrium. If one of them is defoliated the balance is upset, and it is rapidly inhibited by the other one.

A similar experiment (*Experiment 2*) was performed on similarly prepared plants of *Vicia faba*. The slightly larger shoots of three "two-shoot plants" had their leaves removed until the largest remaining leaves were between 0.7 and 1.5 mm. long. During the next 16 days the growth in length of these shoots was only about one-eighth of that of the intact shoots of the same plants, and the growth of their largest remaining leaves was only about one-seventh of that of the corresponding leaves of the intact shoots.

Two similar single shoots, belonging to other plants of the same batch, were similarly defoliated as controls. These grew in length about half as fast as the intact shoots, and their largest remaining leaves grew at about the same rate as the corresponding leaves of those shoots. Thus the result of this experiment was similar to that of the experiment on peas, except that in *Vicia faba* defoliation did distinctly retard the growth in length of the single-control shoots, though not nearly so much as it retarded the shoots of the "two-shoot plants."

In other experiments on "two-shoot" pea plants it was found that when the defoliations were not carried so far, so that the leaves remaining on the defoliated shoots were a little larger than in Experiment 1, though still quite small, then these shoots were not noticeably retarded. This indicated that in Experiments 1 and 2 it was not the removal of the expanded photosynthetic leaves which was effective, but the removal of the unexpanded, rapidly developing leaves. This point is confirmed by the following experiment.

Experiment 3.—A plant of *Vicia faba* was selected with two nearly equal cotyledonary shoots. From the slightly longer shoot the leaves in the stage of rapid development were removed, so that there remained the two oldest leaves, which were expanded and fully-grown, and also the very small leaves near the apex, of which the largest (the seventh leaf) was 1 mm. long. From the other shoot the three oldest leaves were removed, so that there remained

two or three unexpanded rapidly developing leaves, of which the longest was 12 mm. long, and also the very small leaves.

In this way a competition was provided between two shoots, of which the first possessed mature expanded leaves and the second possessed unexpanded rapidly developing leaves, while both possessed also the very small leaves of a length of 1 mm. or less, which have little influence. The largest remaining leaves of the second shoot were continually removed in turn as soon as they began to expand.

After 16 days it was found that the first shoot was 116 mm. long and the second 158 mm. long, both having been about 60 mm. long at the start. The largest of the remaining small leaves of the first shoot was 3.25 mm. long and the corresponding leaf of the second shoot 6 mm. long, both having been 1 mm. long at the start. Thus the second shoot, the one with the unexpanded leaves, had considerably outgrown the shoot with the mature expanded leaves, although it had been very slightly the shorter at the start.

The following experiment also shows that the effect of defoliating a shoot of a "two-shoot" plant is not due to the diminution of its photosynthetic area.

Experiment 4.—This experiment was similar to Experiment 1, except that after the operations the plants were kept in very dim light, in a completely shaded position under the benches of the greenhouse, where photosynthesis can only have been extremely slight, and can hardly have supplied any appreciable amount of nutriment in comparison with the supplies coming from the cotyledons, which lasted throughout the experiment.

Six "two-shoot" pea plants, of race "Sutton's Matchless," were operated upon, one shoot of each being defoliated. In five of the defoliated shoots the largest remaining leaves were the fifth, and were from 0.7 to 0.8 mm. long. In the sixth shoot (that of plant No. 3 of Tables III and IV) the largest remaining leaf was the fourth, and was 1.2 mm. long. The defoliated shoots were slightly the larger in five of the plants, and slightly the smaller in the sixth (No. 4 of the tables).

Three similar single cotyledonary shoots, belonging to plants of the same batch, were defoliated as controls, being left each as the only shoot on its plant. In these, the largest remaining leaves were from 0.7 to 0.8 mm. long.

The growth of the shoots during the first 15 days and the rate at which they developed their leaves is shown in the following tables:—

Table III. Experiment 4. *Pisum sativum*.—Growth of Shoots in millimetres in Dim Light.

"Two-shoot plants."		Single-shoot controls.
Six defoliated shoots.	Six intact shoots.	Three defoliated shoots.
16	72	41
8	55	51
4.5	53	50
11	76	—
11.5	67	—
32	65	—

Table IV. Experiment 4.—Growth in millimetres of largest remaining Leaves of Defoliated Shoots, and of corresponding Leaves of Intact Shoots in Dim Light in 15 days.

"Two-shoot plants."		Single-shoot controls.
Six defoliated shoots.	Six intact shoots.	Three defoliated shoots.
2.0	9.25	5.25
0.5	3.25	6.25
1.3	9.75	11.25
0.5	5.25	—
0.75	6.25	—
6.5	7.75	—

From these tables it can be seen that in the first five of the six "two-shoot plants" the defoliated shoots were enormously retarded both in their growth in length and in the rate at which they developed their leaves, just as they were in Experiments 1 and 2. Only in the sixth plant was this retardation, for some reason, only very slight. Meanwhile, the defoliated single control shoots developed their leaves at about the same rate as the intact shoots, and grew in length only slightly less rapidly.

At 15 days after the operations the plants were placed in good light again. The defoliated shoots of the first five "two-shoot" plants grew no more, but gradually turned yellow, and at 26 days they were dying, whereas the intact shoots of the same plants were healthy and growing.

The results of this experiment were thus similar to those of Experiments

1 and 2: the defoliated shoots of all but one of the "two-shoot" plants were arrested in growth, and finally killed. This must again have been because defoliation somehow caused them to be inhibited by the intact shoots, for the defoliated single control shoots suffered no such harmful effects. Moreover, since the defoliated shoots of "two-shoot" plants are arrested about as rapidly when the whole plants are kept in very dim light as when they are in good light, it follows that in the main it is not the diminution of their photosynthetic area that causes them to be inhibited by the intact shoots. On the contrary, from the results of Experiments 3 and 4, and also from the fact that the defoliations are often not effective unless they are carried right up to the leaves of very small size, it seems sufficiently clear that it is the loss of the rapidly developing unexpanded leaves that causes these shoots to be so rapidly inhibited.

The following experiment shows another way in which the balance between two shoots can be upset, though not so rapidly as by defoliating one of them.

Experiment 5.—Six "two-shoot" pea plants, of race "Sutton's Matchless," were selected, in which the cotyledonary shoots were nearly or quite equal. When they were still small, having expanded not more than one leaf each, one shoot of each plant (the larger shoot, if they were unequal) was covered over with a cylinder of black paper, closed at the top, so that it was almost completely in the dark. Six nearly similar single cotyledonary shoots, belonging to other plants of the same batch, were similarly covered over as controls, being left as the only shoots on their plants.

After 12 days the shoots and young leaves were measured, except those of the first of the "two-shoot" plants and the first of the controls, which were measured after 11 days. The rates at which the shoots developed their leaves can conveniently be expressed in terms of the number of "plastochrons" through which they passed in these times, the word "plastochron" being here used to denote the period taken by a young leaf to grow to the size that was possessed by the next larger leaf at the beginning of the period. The results are given in Table V.

The table shows that in the first 12 or 11 days the darkened shoots of the "two-shoot" plants grew in length only about half as much as the illuminated shoots of the same plants, and developed their leaves only about one-third as fast. But the darkened single control shoots grew in length distinctly faster than the illuminated shoots (this being the usual result of etiolation), and developed their leaves only a little less rapidly.

Table V. Experiment 5. *Pisum sativum*.—Growth of Shoots in millimetres, and Development of Leaves in "Plastochrons" (P) in 12 or 11 days.

Six "two-shoot" plants.		Six single control shoots
Darkened shoots.	Shoots in light.	Darkened.
10 mm. and 0.5 "P"	29 mm. and 2.0 "P"	28 mm. and 2.1 "P"
15 mm. and 0.15 "P"	24 mm. and 1.6 "P"	33 mm. and 1.1 "P"
25 mm. and 0.75 "P"	30 mm. and 2.0 "P"	50 mm. and 1.75 "P"
13 mm. and 1.0 "P"	27 mm. and 2.5 "P"	29 mm. and 1.3 "P"
15 mm. and 1.0 "P"	30 mm. and 2.1 "P"	51 mm. and 2.1 "P"
25 mm. and 1.0 "P"	41 mm. and 2.2 "P"	37 mm. and 1.5 "P"
Means 17.1 mm. and 0.73 "P"	30.1 mm. and 2.07 "P"	41.3 mm. and 1.64 "P"

After the first 12 or 11 days, all the darkened shoots were uncovered and exposed to light, but in spite of this the previously darkened shoots of the "two-shoot" plants grew on only very slowly, or not at all. Their mean growth in length in the next eight days was only 3.2 mm., and their mean development of leaves 0.3 plastochrons, whereas the corresponding figures for the previously illuminated shoots for the same period were 25.6 mm. and 1.66 plastochrons, and for the previously darkened single shoots 22.3 mm. and 1.52 plastochrons. Two days later, or 22 days after the beginning of the experiment, most of the previously darkened shoots of the "two-shoot" plants were just beginning to die, although in some of the plants to which they belonged the cotyledons were not yet exhausted. Usually the expanded leaves of these shoots turned yellow and died first, then the very small leaves of the apical bud did the same, and finally the stems turned yellow and died from the apex downwards. On the other hand, the previously illuminated shoots of the same plants and the previously darkened single control shoots all remained healthy and grew on indefinitely.

From these results it can be seen that the effect of darkening one shoot of a "two-shoot" plant was similar to the effect of defoliating one shoot, though less sudden. The darkened shoots of these plants were strongly retarded, and finally arrested and killed, although after 11 or 12 days they were exposed to light again. This must have been because they were inhibited by the other shoots, for the darkened single control shoots were only slightly retarded in the development of their leaves and were accelerated in their rate of elongation.

The darkening of one shoot of a "two-shoot" plant must therefore somehow upset the balance between the shoots, just as defoliation does, and so lead to the

inhibition of the darkened shoot. But the exact way in which it does so needs to be further investigated. It is possible that it is by slightly retarding the rate of development of leaves that darkening upsets the balance, and so causes the darkened shoot to be inhibited. For it is well known that in darkened shoots the young leaves do regularly develop less rapidly, and it can be seen from the tables that actually they did develop a little less rapidly in the darkened single control shoots than in the illuminated shoots of the "two-shoot" plants. But it is also possible that the lack of light may, in some more direct way, make the darkened shoot more susceptible to inhibition.

Discussion and a Further Experiment.

It may now be considered what light the experiments throw upon the question propounded at the beginning of this paper; how it is that two cotyledonary shoots do not inhibit one another, or not more than slightly, provided that they are about equal in size. The experiments have shown that if one of the shoots is defoliated or darkened, then the other shoot does inhibit it.

The most suggestive point in the defoliation experiments is that it appears to be the removal of the *rapidly developing* young leaves which causes the defoliated shoot to be inhibited; for these leaves are the members of the shoot which tend to inhibit axillary buds or other shoots. From the present experiments it appears that these leaves also protect their own shoot from being inhibited, since when they are removed the shoot to which they previously belonged is rapidly inhibited by another intact shoot, if one is present.

The developing leaves thus tend to bring about two effects: they tend to inhibit other buds or shoots, and they also tend to protect their own shoot from inhibition. Now it is possible that these two effects are due to two quite different influences coming from the developing leaves. Nevertheless it seems much more probable that they are both brought about by the same influence transmitted from the leaves, but that this influence is somehow of a "polar" nature, so that it counteracts another similar influence coming from the opposite direction; for if this is so, it becomes possible to explain the results in the following way without postulating two influences of different kinds. So long as two cotyledonary shoots are growing equally, the influences transmitted from their developing leaves pass down the shoots, and when they meet they counteract one another, since they are coming from opposite directions. The shoots therefore go on growing; but if one of the shoots is defoliated or weakened by darkening, then the influence coming from the developing leaves

of the other shoot travels across into the defoliated or darkened shoot and inhibits it.

But as soon as the suggested explanation is stated in this way, it becomes clear that though it offers an answer to the question how it is that two equal cotyledonary shoots do not inhibit one another, there remains a second equally urgent question. This is the question how it is that the defoliated or darkened shoot is inhibited and finally killed (directly or indirectly) by the influence coming from the developing leaves of the other shoot, although that other shoot is not itself inhibited or harmed by the same influence. In considering this question, it is necessary to consider separately the arrest and final killing of the growing parts, and the killing of the fully-grown parts. As far as concerns the killing of the fully-grown parts, it appears that the difference between the fates of the two shoots is somehow due to their being differently orientated with regard to the influence coming from the developing leaves; for the lower parts of the intact shoot, which remain healthy, are in the line between the developing leaves and the roots, and the influence coming from these leaves travels downwards through these parts basipetally. But the defoliated shoot, which is killed, is not in this line, and in it the same influence travels acropetally.

That the explanation must be sought along these lines seems to be made clear by the following simple experiment (Experiment 6), which was repeated many times. Pea seedlings that had elongated several internodes were decapitated in an upper internode, and were also defoliated, though this was probably unnecessary. Two axillary buds were left on the main shoot, and usually one of these took the lead and went on growing alone to form a new shoot. Above the node at which the new axillary shoot originated, there remained two or three internodes of the decapitated main stem, and after about a month these turned yellow and died from the top downwards to a level from 5 to 10 mm. above the origin of the axillary shoot. The remaining short length of from 5 to 10 mm. immediately above the insertion of the axillary shoot remained alive for much longer. In several plants it was still green and healthy after about two months, but after a still longer time usually most of this remaining length died also. But the part of the main stem below the axillary shoot remained green and healthy indefinitely, as was indeed to be expected, since it served to connect the growing axillary shoot with the roots.

There is therefore no occasion to postulate that the main stem possessed any special kind of susceptibility to inhibition different from that possessed by the axillary shoot; for the lower part of the main stem, which was in the line

between the developing leaves of the axillary shoot and the roots, and in which the influence coming from those leaves travelled basipetally, remained healthy, as did the axillary shoot itself. It was only the part of the main stem which was not in this line, and in which the influence travelled acropetally, that was killed.

The general rule, therefore, seems to be that those fully-grown parts of shoots which are killed by inhibition are the parts which are out of the line between developing leaves and roots, and in which the influence coming from such leaves travels acropetally. The present results do not indicate which of these two points is the critical one—that is to say, whether the shoots are killed because they are out of the line between developing leaves and roots, or because the leaf-influence travels through them acropetally. But it is intended to investigate this question experimentally, and in so doing to test the hypothesis, mentioned in a previous paper (Snow, 1931), that the influence coming from the developing leaves somehow polarises the stem in such a way that some nutritive or growth-promoting substances tend to flow through it in the opposite direction towards the developing leaves.

It remains to be considered how it is that the growing zones of the defoliated shoots of the "two-shoot" plants were inhibited and finally killed, although those of the intact shoots were not. This question is more complicated than the corresponding question with regard to the killing of the fully-grown parts. For the growing-zones of the two shoots were not only differently orientated with regard to the influence coming from the developing leaves of the intact shoot; they were also situated at very different distances from them. Now in the previous paper of this series it was shown that the intensity with which the developing leaves of a pea shoot inhibit axillary buds below them increases with increasing distance of these buds from them. It was further shown that so long as the axillary buds are within a region quite close below the developing leaves, they are not inhibited at all, or only slightly at most. The length of this region is not more than 15 mm. and probably not less than about 5 mm. This being so, it would in any case hardly be expected that the developing leaves would inhibit the growing zones of their own shoot; for in the pea these zones are fairly close below them. They do inhibit the growing zones of the other shoot (the defoliated shoot), since these are sufficiently distant from them, and they also inhibit axillary buds which are sufficiently distant. But in the defoliated shoot, and also in axillary buds, the influence coming from the developing leaves of the intact shoot is travelling towards the apex. Consequently, it cannot be said without further investigation whether the same

influence, when travelling towards the base, would inhibit any growing zones through which it passed if they were distant from the developing leaves, for in the pea no such zones exist. It is intended to investigate further the effects of the developing leaves on the growth of their own shoot, in comparison with their effects on other buds and shoots.

Summary.

(1) By decapitating young pea seedlings in the epicotyl, plants can be prepared with two shoots springing from the axils of the cotyledons.

(2) In such plants, if one of the shoots has its leaves removed until only those 1 mm. long or less remain, it is rapidly arrested in growth and finally killed.

(3) This effect must be due to inhibition coming from the intact shoot, for if similar *single* shoots are similarly defoliated they grow on rapidly and indefinitely.

(4) It makes no appreciable difference to these results whether the plants are grown in good light or, for the first 12 days after the operation, in very dim light.

(5) From these and other experiments, it is concluded that the effective part of the defoliation is the removal of the rapidly developing young leaves, which are also the inhibiting members, and not the removal of the expanded photosynthetic leaves.

(6) Similar results are produced, though less rapidly, by darkening a shoot. If another shoot is present on the same plant, the darkened shoot is arrested and killed, but if not it grows on rapidly.

(7) These results make it probable that the inhibiting influence that comes from the developing leaves of a shoot is of a polar nature, so that two such influences coming from two cotyledonary shoots from opposite directions counteract one another. If one of the shoots is defoliated or otherwise weakened, the influence coming from the other shoot travels up into it, and arrests and kills it.

(8) The influence coming from the developing leaves kills (directly or indirectly) these shoots or parts of shoots which are not in the line between developing leaves and roots, and in which it travels acropetally.

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Respiration as a Factor in the Ionic Equilibria between Plant Tissues and External Solutions.

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The experimental work was done in the Botany School, Cambridge, while one of us (A. H. K. P.) was an 1851 Exhibition Scholar.

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Many investigations have been made of the salt exchange between slices of plant storage organs and solutions in which they have been placed. A simple form of experimentation is to place the pieces of tissue in water devoid of solutes, and to investigate the drift with time of the concentration of the external solution. The concentration of such a solution was used by Stiles and Kidd (1919) as a base line from which to calculate the absorption of salts by the tissue when placed in a salt solution. For example, the conductance of distilled water rose by 190 units in 52 hours when carrot tissue was placed in it, whereas that of 0.0002 N KCl increased only 137 units, whence it was deduced that the carrot tissue had absorbed KCl to the extent of 53 units of conductance. The justification for this procedure is doubtful, since a solution with which the tissue was in equilibrium would show no change of conductivity, and yet by this method of reasoning would be said to have lost salt to the extent of 190 units of conductance. More recently Stiles (1927) has made detailed studies of the problem of the exosmosis of substances from slices of various storage tissues, including carrot root, into distilled water. Well-washed discs of tissue were placed in water and shaken continuously at 20° C. The drift of the conductance of the outside solution was followed. It rose for about 24 hours and then fell slowly for about 10 days, after which a steady value was maintained for some time until the conductance rose as the tissue died. The concentration of the outside solution was also determined by the weight of the residue after evaporating the solution to dryness. The concentration so determined followed the same course as the conductance.

Stiles suggests that the initial liberation of electrolytes, which is greater the shorter the preliminary washing, takes place from the cut surface-cells and from the cells underneath, which soon die. These electrolytes are absorbed

by the remaining living cells at a rate which is eventually greater than that at which they are liberated, with a consequent fall of the external conductivity. The tissue, small in bulk relative to the solution, would have to absorb the greater part of the electrolytes liberated. There is no direct evidence in favour of such an explanation. As far as we know, tissue, such as Stiles used, never decreases the conductivity of such dilute solutions, except in experiments of the type which we are trying to explain.

There are probably many factors concerned with the distribution of electrolytes between tissue and the surrounding solution. One important factor which has been suggested is the presence of substances such as protein ions, which cannot escape from the tissue. Since the fraction of protein electrolytes which is ionised depends upon the concentration of hydrogen ions, this concentration should play an important part. Unpublished work on this question shows that such is the case. As one of the products of respiration of plant cells, carbon dioxide, is an acid in solution, the rate of respiration will affect the concentration of hydrogen ions, and so, through the degree of ionisation of protein electrolytes, will also affect the distribution of the other electrolytes between the tissue and the surrounding solution. Moreover, the respiration of most plant tissues is much increased as a result of cutting.

Our object has been to investigate the relation between the intensity of respiration, as measured by evolution of carbon dioxide, and the exosmosis of electrolytes.

Experimental Work.

Discs of carrot root, 0.1 cm. thick, 1.75 cm. in diameter, and 80 in number, were placed, after preliminary washing, in a vessel containing 200 c.c. of freshly distilled water maintained at a temperature of 15° C.* A stream of air free from carbon dioxide was passed through the water, and the carbon dioxide evolved determined by absorption with baryta. The vessel contained a pair of platinum electrodes, protected by a glass cage from contact with the tissue. Periodically the current of air was stopped temporarily, and the conductivity of the solution was determined. In preliminary experiments the vessel was shaken, but it was subsequently found that the agitation caused by the stream of air was sufficient to maintain the vitality of the tissue. Without agitation, the conductivity of the outside solution steadily rises as the tissue dies.

The results of a typical experiment are shown in fig. 1. It will be seen that the general trend of the conductivity curve is the same as that in the experi-

* The volume of the solution is about ten times that of the tissue.

ments of Stiles, but what is most noteworthy is the similarity of the changes of conductivity and the rate of evolution of carbon dioxide by the system.

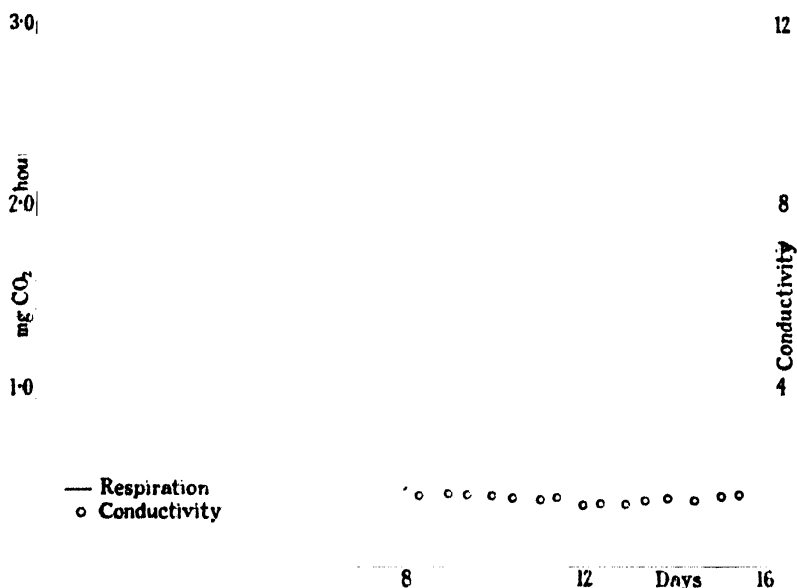


FIG. 1.

With freshly cut tissues the situation is complex ; not only is the rate of respiration higher than normal, but there are other processes at work which Stiles suggests are the main controlling factors—the diffusion of electrolytes from incompletely-washed cells which have been cut, and possibly from dying cells. It is clearly desirable to change the rate of respiration without cutting the tissue, and to investigate the effect of this on the external conductivity. In the second experiment (see fig. 2) the air current was changed for one of pure oxygen on the seventh day, when the rate of respiration and the conductivity appeared to have approached closely to steady values. There was no significant effect on either respiration or conductivity. On the eleventh day the gas current was changed to one of commercial nitrogen (about 0.5 per cent. oxygen) free from carbon dioxide. The rate of respiration and the conductivity were both increased. Then, on the thirteenth day, the current was changed back to air again, when both the rate of production of carbon dioxide and the conductivity fell rapidly.

The effects of a decrease in the oxygen supplied will be more than an increase in the rate of production of carbon dioxide. Products of anaerobic metabolism of sugar, such as alcohol, might change the permeability ; but this

should not affect the final distribution of electrolytes, only the rate at which equilibrium is attained. Some of the cells may be killed and their contents

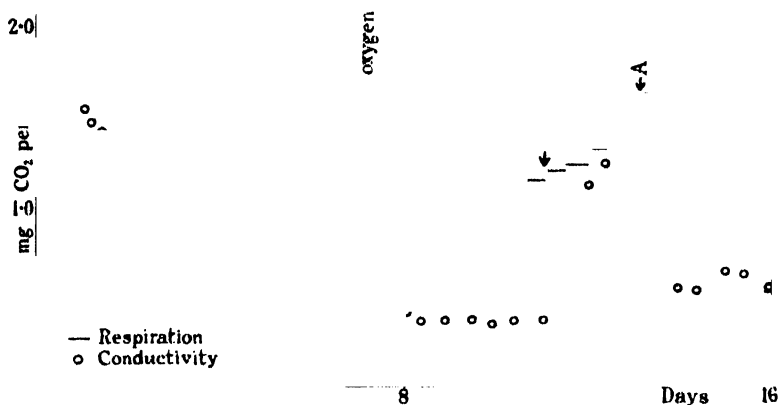


FIG. 2.

pass out into the solution. The sudden fall of the conductivity when the current is changed back to air suggests that death of cells is a negligible factor. Possibly organic acids are produced and pass out into the solution, and are subsequently oxidised to carbon dioxide and removed.

The fact established by these experiments is that there exists a general parallelism between rate of respiration and conductivity of the outside solution. In the next section we shall consider the theoretical basis for such a state of affairs. For this purpose a knowledge of the actual values of the conductivity will be useful. The unit used in the graphs is that of the distilled water into which the tissue was placed—about 1.56×10^{-6} reciprocal ohms. This is the value for a solution of carbon dioxide about five times as concentrated as that of a solution in equilibrium with the atmosphere (0.03 per cent. CO_2) at 18°C . To increase the conductivity to nine units would necessitate about 5.5 per cent. of carbon dioxide in the atmosphere, to 12 units, about 9.5 per cent.

Theoretical Survey of the Effect of Respiration.

§ 1. In considering the effect of a change in the rate of respiration on the distribution of electrolytes between the tissue and the outside solution, we must distinguish two distinct types of effect—the mobility and the ionisation effects. Further, we must distinguish between conductivity and total solids left after evaporation, the two measures used by Stiles for the concentration

of the outside solution. Of course, other substances than electrolytes may contribute to total solids.

§ 2. To take the last point first : the total concentration of ions may remain unchanged despite changes in both conductivity and total solids. Moreover, the changes in conductivity and total solids may not agree in direction. If from an outside solution of KCl some of the K ions are taken up by the tissue and their places taken by H ions, the conductivity of the solution would be increased because of the greater mobility of the H ions, yet the total ionic concentration would be the same. At the same time, the total solids would be decreased, since the HCl would be lost on evaporation. Replacement of the Cl ions of the solution by OH ions would likewise increase the conductivity, and again the total solids would decrease by the substitution of KOH for KCl in the residue from evaporation.

§ 3. If the mobilities* of the two ions of carbonic acid produced by respiration in the tissue are different, then the outward diffusion of the carbon dioxide would result in a potential gradient along its path. If the hydrogen ion has the greater mobility, the outside solution will tend to be positive to the tissue and diffusible cations, such as K, will tend to be in greater concentration in the tissue than outside, whilst diffusible anions, such as Cl, will tend to be in smaller concentration in the tissue. The excess of diffusible cations over anions in the tissue will balance the excess of slowly moving HCO_3 ions over H ions in the tissue. If the HCO_3 ion has the greater mobility, then the concentration of diffusible anions will tend to exceed that of the diffusible cations in the tissue. An increase of the rate of respiration would tend to cause an uptake of ions such as K and a loss of ions such as Cl by the tissue, if the H ions had the greater mobility, a loss of K ions and an uptake of Cl ions if the HCO_3 ions had the greater mobility.

§ 4. In the cytoplasm there are substances, some of a protein nature, whose degree of ionisation depends upon the concentration of hydrogen ions. If the concentration of hydrogen ions is such that the non-diffusible anions derived from these weak electrolytes predominate over the non-diffusible cations, then there will be more diffusible cations, such as K, than anions, such as Cl, in the cytoplasm, the reverse being the case if the non-diffusible cations predominate. An increase in the rate of respiration will entail a series of consequences. The concentration of hydrogen ions in the cytoplasm will tend to increase. Such an increase will depress the ionisation of the weak

* It is the mobility of the ions in the medium through which they pass as they come out of the tissue, not that in water, with which we are concerned.

electrolytes of an acidic nature, and increase the ionisation of those of a basic nature—in other words, the non-diffusible anions will be decreased and the non-diffusible cations increased. This will result in an uptake by the tissue of diffusible anions and a loss of diffusible cations.

§ 5. The mobility and ionisation effects will work together if the mobility of the HCO_3 ion exceeds that of the H ion. If the reverse holds it will depend upon which effect is greater as to whether an increase in the rate of respiration will result in an uptake of anions, such as Cl, and a loss of cations, such as K, by the tissue, or a loss of anions and an uptake of cations. Analyses of the outside solution are necessary before we can say which type of ion, anion or cation is taken up, and which is lost by the tissue, when the rate of respiration increases.

§ 6. The effect of the ionic interchange on the conductivity of the outside solution must now be considered. If the gas current, which is passing into the solution, is practically devoid of carbon dioxide (as in our experiments), then, when the rate of evolution of carbon dioxide is doubled, the concentration of undissociated carbonic acid in the solution close to the gas phase is also doubled. The product of the concentrations of H and HCO_3 ions must also be doubled. If there were no other ions present in the outside solution, then the concentrations of the H and HCO_3 ions would each be increased $\sqrt{2}$ times, and hence the conductivity would be increased $\sqrt{2}$ times when the rate of evolution of carbon dioxide was doubled. The presence of ions such as K and Cl makes the question more complicated. With our limited knowledge of the details of the system with which we are dealing, we can only outline some of the possibilities. In the following table are the relative values for the conductivity of a solution containing carbon dioxide with different concentrations of K ions.

Conductivity of Solutions of Carbon Dioxide containing KHCO_3 .

Concentration of K.	$(\text{H}_2\text{CO}_3 + \text{CO}_2) = 1.25 \times 10^{-3}$.		$(\text{H}_2\text{CO}_3 + \text{CO}_2) = 2.5 \times 10^{-3}$.	
	<i>pH</i> .	Relative Conductivity.	<i>pH</i> .	Relative Conductivity.
0	5.71	1	5.56	1.4
10^{-3}	6.43	1.7	6.15	1.9
2×10^{-3}	6.73	3.1	6.43	3.2
4×10^{-3}	7.03	6.1	6.73	6.1
8×10^{-3}	7.36	12.2	7.03	12.2

Two different concentrations of carbon dioxide have been used, one containing as much undissociated carbonic acid as it would have when in equilibrium with an atmosphere containing 0.03 per cent. CO_2 at 18°C ., and the other twice as much. The concentration of H ions is given in the form of p_{H} . The calculations are based on the data of Walker and Cormack (1900). When the respiration of the tissue had approached a more or less steady state after its initial high value, the conductivity of the external solution was about seven times that of a solution of carbon dioxide in equilibrium, with the atmosphere containing 0.03 per cent. CO_2 . Such a conductivity would be given by a solution containing about 4.5×10^{-5} grm. ions of K.* This holds whether the undissociated carbonic acid is that in equilibrium with an atmosphere containing 0.06 per cent., 0.03 per cent. or less of carbon dioxide. If it were 0.0003 per cent. it would mean a reduction of the conductivity of less than 1 per cent. Under such conditions a doubling of the rate of evolution of carbon dioxide with a consequent doubling of the concentration of undissociated carbonic acid would have no appreciable effect on the conductivity unless the concentration of K ions was changed. When the concentration of carbon dioxide in the tissue is increased there is a tendency for the concentration of the H ions in the tissue to increase, but this is balanced more or less completely by the depression of the ionisation of the weak acids (or the increased ionisation of the weak bases) of the tissue, with the result that cations, such as K, pass out into the solution. For the time being we are neglecting the mobility effect. If the weak electrolytes are in sufficient concentration, a doubling of the rate of respiration will result in no appreciable change of the concentration of H ions, in the tissue or in the external solution. In that case, a doubling of the concentration of undissociated carbonic acid in the external solution would necessitate the concentrations of K and HCO_3 ions being almost doubled. As the H ion contributes a negligible fraction of the conductivity, this quantity also would be almost doubled. This proportionality of conductivity and concentration of undissociated carbonic acid, or rate of evolution of carbon dioxide, would hold fairly closely over a yet wider range of variation of carbonic acid concentration. The above argument allows for the presence of cations, such as K. If diffusible anions, such as Cl, were present in addition, when the cations came out of the tissue the anions would go in, and the increase in conductivity of the external solution would not be so great. At another time we propose to consider in detail the possibility that normally the cell sap can exchange anions, but not cations, with the cytoplasm and the external

* A greater concentration of a less mobile cation, such as Na, would be required.

solution. If such is the case the increase in concentration of anions, such as Cl, in the cytoplasm may be mainly at the expense of the sap, HCO_3 ions taking the place of the Cl ions. The increase in the HCO_3 ions in the sap may actually be great enough to displace some of the sap anions into the outside solution. We have unpublished data showing a check in the course of uptake, sometimes an output, of both anions and cations by carrot tissue from a salt solution at the time when the respiration of the tissue is about its maximum. It does not seem profitable to attempt an exploration of further possibilities until we know more about the system with which we are dealing.

§ 7. Although there is a general parallelism of the rate of evolution of carbon dioxide and conductivity, yet it will be seen in fig. 2 that when the rate of evolution of carbon dioxide increased, consequent upon the reduction of the oxygen supply, the percentage increase of the conductivity was greater than that of the rate of evolution of carbon dioxide. There probably are factors other than those taken into account in our theoretical considerations—factors such as the anærobic breakdown of sugars—which might contribute to this extra increase in conductivity. There is, however, a further factor, external to the respiring tissue, which needs consideration—a complication inherent in all attempts to measure rate of carbon dioxide production by rate of evolution of this gas.

§ 8. The rate of evolution of the carbon dioxide into the gas current devoid of carbon dioxide depends upon the concentration of this substance in the liquid immediately outside the bubble of the gas current. If the liquid were maintained in a state of great agitation, the concentration of carbon dioxide would be uniform throughout the liquid. Actually, only a slow stream of gas was taken through the solution, so that the liquid immediately outside the tissue would be richer in carbon dioxide than that close to the gas bubble. A doubling of the rate of respiration would result, at first, in a greater increase in the concentration of the carbon dioxide around the tissue than around the bubble. Eventually, the latter also would be doubled if the new rate of respiration was maintained; but if, before this state was reached, the rate of respiration fell again to its original value, the concentration of carbon dioxide around the bubble would never attain the double value. Actually, a rise and fall in the rate of production of carbon dioxide by the tissue, giving an extra amount of this gas, would be followed by a rise and fall of the rate of evolution of carbon dioxide by the solution, giving the same extra amount of the gas; but the rate of evolution would never rise as high as did the rate of respiration. The phase of increased evolution by the solution would last longer than the

phase of increased production by the tissue. The conductivity was measured in the bulk of the liquid, nearer to the tissue than was the stream of gas, and was more closely related to the rate of respiration of the tissue than to the rate of evolution of carbon dioxide by the solution. The changes of conductivity should, therefore, be greater than the changes of rate of evolution of carbon dioxide, taken as measures of respiration, would lead us to expect. Actually, the results do bear out the above suggestions: the conductivity values do, on the whole, rise and fall before those for the rate of evolution of carbon dioxide.

§ 9. There remains to be considered the question as to the change of the concentration of the total solids in the external solution consequent upon an increase in the rate of respiration. If the outside solution contains such substances as KHCO_3 and KCl , as we have suggested, an increase in the concentration of HCO_3 , accompanied by an increase in the concentration of K and a decrease in the concentration of Cl ions, would be equivalent to an addition of KHCO_3 and a substitution of KHCO_3 for KCl in the total solids. In this way the total solids would increase with the rate of respiration; for heavier negative ions, such as SO_4 and PO_4 , and for sodium, a metal of smaller atomic weight, the increase of total solids might be less. A loss of cations and a gain of anions by the outside solution when the respiration increased would be likely to result in a decrease of the total solids, since the salt content would be decreased and the increased content of acids such as H_2CO_3 , HCl and H_2SO_4 would not appear in the total solids left on evaporation.

§ 10. Elsewhere one of us (Briggs, 1930) has suggested that the accumulation of cations and anions in the sap of some plant cells (not in the cell as a whole) could be accounted for by alternating phases of permeability of the cytoplasmic membrane enclosing the sap; one phase where the membrane is more permeable to cations and the other more permeable to anions. Such an accumulation of ions is increased by an increased rate of respiration. The *schema* considered in the present paper for mature cells assumes that this accumulation of ions in the sap is no longer appreciable, and that the effect of change of respiration on the cytoplasm itself predominates in deciding the ionic interchange between the cell, as a whole, and the external solution.

We have attempted to show that the rate of respiration is an important factor deciding the concentration of electrolytes in water into which pieces of plant tissue have been placed. The experiments are only of a preliminary nature; much remains to be done before we have a full knowledge of the system with which we have been dealing. Till then we cannot evaluate the

relative importance of such factors as leaching of dead and dying cells with subsequent absorption by living cells which, till the present, have alone been invoked to explain the phenomena.

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Further Investigation of Sweating and Sweat.

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Various points requiring further investigation were left over from the paper communicated in 1929 (1), and the present paper describes the further study of these questions. A description of the methods employed, including analysis, will be found in the previous paper.

The subject of the experiments was a miner from a colliery in North Staffordshire, who had been more or less acclimatised to working in a hot place, although lately he had been employed on surface work for some months. The experiments were carried out in two groups of 1 week each, one of a fortnight, and a further one of 1 week, the results being shown in Tables I, II, III, IV and V.

Tabl

Experi- ment No. (Period of 1 hour).	Date, September, 1920.	Average air temperature in chamber.		Final mouth tempera- ture.	Final pulse rate, resting.	Total weight lost through the skin.	Total chlorine.	Chlorine Potassium.	Cl calculated as KCl $\frac{\text{Total Cl}}{\text{Total CL}} \times 100$.	Rate of work.	Water drunk during experi- ment.
		Dry- bulb.	Wet- bulb.								
20 (a) (b) (c)	16	90.3 91.9 93.4	89.1 90.1 93.1	99.1 99.5 100.2	76 88 124	grams 60 160 245	grams 0.093 0.097 0.162	per cent. 0.105 0.061 0.066	— — —	ft.-lb./m. nil " "	grams nil 640 nil
		95.5 93.8 94.2 94.5	93.7 93.2 94.2 94.5	99.2 99.0 100.0 100.6	96 96 124 140	grams 615 505 230 110	grams 0.668 0.728 0.239 0.163	per cent. 0.109 0.144 0.104 0.148	— — — —	nil " " "	grams nil 1305 340 396
		93.4 94.0 93.2	87.9 89.8 89.7	99.0 100.2 101.4	92 124 140	grams 695 745 655	grams 0.328 0.869 0.479	per cent. 0.047 0.117 0.073	37 15 17	1550 1940 2355	nil 595 1120
23 (a) (b) (c)	19	93.6 93.3 92.4	89.0 91.8 89.8	99.2 99.9 100.5	104 96 128	grams 1080 380 490	grams 0.753 0.271 0.294	per cent. 0.070 0.071 0.060	25 28 27	1615 nil 1475	395 610 580
		92.5 93.1 91.8	86.7 85.3 86.8	99.0 99.6 99.8	92 104 128	grams 1055 725 415	grams 0.610 0.515 0.423	per cent. 0.058 0.071 0.102	30 26 23	2290 2825 2350	405 610 930

Table II.

Experi- ment No. (Period of 1 hour).	Date, October, 1929.	Average air temperature in chamber.		Wet Kata cooling power.	Final mouth tempera- ture.	Final pulse rate, resting.	Total weight lost through the skin.	Total chlorine.	Chlorine, per cent.	Potas- sium, per cent.	Cl calculated as $\frac{\text{KCl}}{\text{Total Cl}} \times 100$.	Rate of work.	Water drunk during experi- ment.
		Dry- bulb.	Wet- bulb.										
25 (a) (b) (c)	23	°F. 92.6	°F. 84.4	—	°F. 98.8	80	grams 370	grams 0.146	per cent. 0.039	per cent. 0.022	49	ft.-lb. km. 1330	grams 500
		93.1	86.4	—	99.0	92	230	0.204	0.089	0.033	33	1540	500
		93.2	85.5	—	99.2	94	240	0.242	0.101	0.034	31	1500	500
26 (a) (b) (c)	23	92.9	84.6	—	98.7	88	340	0.193	0.067	0.028	44	1280	500
		93.5	85.3	—	98.7	88	296	0.212	0.072	0.026	33	1630	500
		93.3	87.1	4.5	99.0	100	275	0.219	0.080	0.027	31	1630	500
27 (a) (b) (c)	24	94.2	85.2	—	99.4	96	430	0.244	0.067	0.030	49	1400	500
		97.7	86.4	—	99.0	92	425	0.319	0.075	0.027	33	1660	500
		94.5	85.4	—	99.2	100	300	0.213	0.071	0.027	35	1530	500
28 (a) (b) (c)	25	92.6	85.6	—	99.0	88	630	0.429	0.068	0.020	27	1620	500
		94.4	87.3	—	99.5	100	406	0.279	0.069	0.019	25	1490	500
		95.0	85.6	—	99.7	92	400	0.203	0.051	0.019	35	1545	500

Tabl

Experi- ment No. (Period of 1 hour).	Date, January, 1930.	Average air temperature in chamber.		Wet Kata cooling power.	Final mouth tempera- ture.	Final pulse rate, resting.	Total weight lost through the skin.	Total chlorine.	Chlorine. per cent.	Potas- sium. per cent.	Cl calculated as $\frac{\text{KCl}}{\text{Total Cl}} \times 100$.	Rate of work.	Water drunk during experi- ment.
		Dry- bulb.	Wet- bulb.										
29 (a) (b) (c)	27	°F. 93.4	°F. 86.2	—	°F. 98.9	76	grams 335	grams 0.153	per cent. 0.045	per cent. 0.029	57	ft.-lb./m. 1585	500
		94.4	86.0	—	98.5	80	290	0.170	0.059	0.028	43	1690	500
		94.3	88.3	—	99.4	100	305	0.210	0.069	0.027	35	1910	500
30 (a) (b) (c)	28	95.3	86.5	—	98.6	80	450	0.272	0.060	0.025	38	1950	500
		95.8	86.5	—	98.7	92	455	0.248	0.055	0.021	34	2045	500
		95.6	87.6	—	99.5	108	375	0.303	0.081	0.024	27	2000	500
31 (a) (b) (c)	29	94.0	86.9	5.3	98.7	80	510	0.255	0.050	0.026	47	1905	500
		93.8	87.5	—	98.9	96	570	0.182	0.032	0.014	40	1735	500
		94.4	87.8	4.2	99.5	104	295	0.184	0.062	0.028	41	1935	500
32 (a) (b) (c)	30	95.8	86.1	—	98.8	84	460	0.267	0.058	0.025	40	1885	500
		95.0	85.3	—	99.0	88	375	0.254	0.068	0.025	33	1945	500
		96.5	87.1	—	99.5	102	345	0.196	0.057	0.024	38	1730	500
33 (a) (b) (c)	31	95.1	88.1	—	99.4	96	785	0.592	0.075	0.023	27	1445	500
		96.5	89.3	2.9	99.9	108	385	0.364	0.095	0.024	23	1850	500
		95.3	91.0	2.4	101.0	120	285	0.246	0.086	0.026	28	1735	500

Table IV.

Experi- ment No. (Period of 1 hour).	Date, February, 1930.	Average air temperature in chamber.		Wet Kata cooling power.	Final mouth tempera- ture.	Final pulse rate, resting.	Total weight lost through the skin.	Total chlorine.	Chlorine, percent.	Potas- sium, percent.	Cl calculated as KCl $\frac{\text{Total Cl}}{\text{Total Cl}} \times 100$.	Average rate of work.	Water drunk during experi- ment.
		Dry- bulb.	Wet- bulb.										
34 (a) (b) (c)	3	°F. 93.6	°F. 87.4	—	°F. 99.3	100	grams 990	grams 1.467	percent. 0.148	percent. 0.026	16	ft.-lb./m. 1895	grams 500
		95.0	88.6	—	99.6	108	615	0.843	0.137	0.021	14	1740	500
		93.5	89.4	—	100.1	120	220	0.320	0.145	0.027	17	1415	500
35 (a) (b) (c)	4	°F. 95.7	°F. 89.4	—	°F. 99.3	90	705	0.773	0.110	0.022	18	1415	500
		96.0	89.2	—	99.6	96	390	0.591	0.152	0.025	15	1290	500
		93.0	90.5	—	99.9	106	205	0.237	0.116	0.031	24	1140	500
36 (a) (b) (c)	5	°F. 95.9	°F. 89.8	—	°F. 99.3	86	780	0.723	0.093	0.022	21	1375	500
		94.9	89.4	—	99.6	96	340	0.438	0.129	0.024	17	1245	500
		94.4	90.7	2.8	100.2	108	370	0.316	0.085	0.019	20	1490	500
37 (a) (b) (c)	6	°F. 95.2	°F. 89.2	3.6	°F. 99.2	84	515	0.466	0.090	0.028	28	1280	500
		97.2	90.1	3.0	99.4	92	465	0.506	0.109	0.023	19	1510	500
		95.0	90.7	—	100.2	102	305	0.312	0.102	0.023	20	1275	500
38 (a) (b) (c)	7	°F. 94.1	°F. 93.6	—	°F. 99.4	88	670	0.563	0.084	0.025	27	nil	500
		94.1	94.1	<0.5	99.3	92	230	0.232	0.101	0.027	24	"	500
		94.4	94.4	<0.5	99.5	104	110	0.154	0.140	0.038	25	"	500

Table V.

Experi- ment No. (Period of 1 hour).	Date, July, 1930.	Average air temperature in chamber.		Wet Kata cooling power.	Final mouth tempera- ture.	Final pulse rate, resting.	Total weight lost through the skin.	Total chlorine.	Chlorine.	Average rate of work.	Water drunk during experi- ment.
		Dry- bulb.	°F. Wet- bulb.								
39 (a) (b) (c)	21	93.3	92.9	1.5	99.1	88	grams 190	grams 0.149	per cent. 0.078	ft.-lb./min. nil	grams 300
		93.7	93.7	0.6	99.1	96	80	0.080	0.075	"	300
		94.7	94.7	—	99.3	104	60	0.054	0.108	"	nil
40 (a) (b) (c)	22	96.4	88.5	3.6	99.2	96	380	0.488	0.128	1660	500
		100.3	91.0	2.6	99.6	96	370	0.513	0.139	1315	300
		102.0	91.8	2.4	100.9	116	285	0.340	0.119	1160	300
41 (a) (b) (c)	23	94.4	94.4	—	98.7	100	75	0.077	0.103	nil	300
		94.3	94.3	—	98.8	100	100	0.086	0.086	"	300
		95.0	95.0	—	99.0	110	75	0.057	0.076	"	300
42 (a) (b) (c)	24	96.8	91.0	2.8	99.3	90	680	0.637	0.094	1455	500
		96.5	91.0	2.8	99.9	102	550	0.614	0.112	1545	300
		99.8	91.2	2.6	100.3	112	285	0.307	0.108	1475	300
43 (a) (b) (c)	25	96.8	96.8	—	99.1	106	230	0.174	0.079	nil	300
		96.6	96.6	—	99.3	112	170	0.209	0.123	"	300
		96.0	96.0	—	99.7	124	110	0.125	0.114	"	300

I.—Effects of Rise of Body Temperature and of Muscular Work on the Amount of Sweat Excreted.

It was found previously that, with the subject at rest, sweating became progressively less copious with an increase in the duration of the sweating even when water was being drunk. This is confirmed in general by the present experiments, and in experiment 21(d) the weight of sweat excreted fell to only 110 grams per hour. With the addition of the water evaporated from the lungs, the total amount of water that could be evaporated per hour would be approximately 115 grams, corresponding to an absorption of 67 large calories per hour. A man at rest produces about 72 large calories per hour (Martin, 1930), so that the whole of the 115 grams of water would require to be evaporated to prevent a rise of body temperature, if no loss of heat occurred by radiation and convection.

The temperature of the walls of the experimental chamber was approximately 4° F. below that of the air in the chamber, and this would increase the loss by radiation and cause convection currents, which would add to the motion of the air caused by the blowing in of heated air through the ventilating pipe. The volume of the experimental chamber was 350 cubic feet and the fan delivered a current of air of the order of 80 cubic feet a minute.

In some of the later experiments (Table V) the loss in weight per hour was only 75 grams, with no progressive rise of body temperature, so that it would appear that radiation and conduction must account for at least one-third of the heat-loss in these cases.

When the subject was performing muscular work the progressive decrease in weight of sweat excreted was much less marked. It will be seen in Table II that with only a moderately high wet-bulb temperature (about 85°) and with the rate of work and amount of water drunk kept as uniform as possible, there was no large difference in either the chlorine concentration of the sweat or in the amount of sweat lost during each period of an experiment. The rise in body temperature was as distinct as in the resting experiments, and yet a moderate sweat excretion was maintained. The increase in the excretion of sweat during the week, combined with a slight decrease in chlorine concentration, indicates that the subject was becoming more acclimatised. In some cases, however, when work was being performed, the amount of sweat lost was large in the first period of each experiment and fell to a considerably lower figure in the last period, although the wet-bulb temperature was gradually increased during the course of the test. This was particularly shown in

experiment 34 and to a smaller extent in experiments 33, 35 and 36, also 23 and 24. A much smaller sweat loss will be seen in the final periods of experiments in which the subject was at rest, although in all cases the subject had plenty of water to drink. This is in accord with previous results, and confirms the conclusion that the sweat-glands are liable to partial exhaustion with prolonged sweating, although during muscular work the exhaustion is much less marked.

Another factor, no doubt, is the degree of acclimatisation of the subject, or his physical condition at the particular time. Thus experiment 33 was performed on the Friday of one week and experiment 34 on the Monday following, and although the wet-bulb temperature was slightly higher in the former, the latter showed a greater sweat excretion, the average rates of work being practically identical.

It was noticeable throughout the experiments that the subject in general seemed more comfortable when working than when at rest, although from the mouth temperature and pulse rate the reverse would rather be expected. This may be largely a psychological matter, however, since merely sitting still in the experimental chamber for hours on end is rather depressing. In hot weather many persons seem to experience less discomfort in taking some kind of active exercise than in sitting down, provided the exercise is not too strenuous. In experiment 22 the rate of work was too high for the subject to prevent a distinct and progressive rise in body temperature.

From the experimental data, it seems that the amount of sweating is not only due to a rise in body temperature, for with the same rise in body temperature as at rest the performance of muscular work considerably facilitates the sweating. It seems probable that some product of muscular metabolism is responsible for this, though the connection might be some less direct one.

II.—The Concentration of the Chemical Constituents of Sweat.

The concentration of chlorine throughout the experiments was lower than that found for previous subjects. It seems, therefore, that J. D. S. would be less likely to suffer from water poisoning than an individual with a higher chlorine concentration of his sweat. It will be apparent that when the subject was at rest the chlorine concentration of the sweat tended to increase in general during the course of each experiment, this being in accord with the results published previously. When the subject was at work this progressive increase was not as marked. The highest concentration sometimes occurred during the second period; but the chlorine concentrations of each period of the

experiment did not vary one from another to the same extent as when the subject was at rest.

It is important to remember that blood-plasma contains about 0·6 per cent. of sodium chloride, while the chlorine concentration of the sweat in these experiments was on the average about 0·08 per cent., corresponding to 0·13 per cent. of sodium chloride. Haldane (1928) has explained very simply that isotonicity in two solutions with the same solvent signifies equality, in the two solutions, of the mutual diffusion pressures of the solvent. The diffusion pressure of solvent (*viz.*, water) is greater in sweat than in blood, so that sweat is strongly hypotonic and must, therefore, be regarded as a very active secretion. With fatigue of the sweat-glands the chlorine concentration rises, as might be expected. Sweat would require to have a concentration of nearly 1 per cent. of sodium chloride, or 0·61 per cent. of chlorine, in order to be isotonic with blood-plasma.

Individual variations with regard to chloride concentration of the sweat and rate of sweating undoubtedly occur, this being borne out by the fact that some persons living in the tropics consume large amounts of water during the day with no ill effects, whilst others either find it essential to drink sparingly or feel no desire to drink copiously. Miners working under exactly similar conditions may drink from 3 pints of water each to 8 pints per shift—although the loss of chloride from the body may be roughly the same in each case through differences in the amount and salt concentration of the sweat excreted.

The effect of raising the wet-bulb temperature of the air on the concentration of chlorine in the sweat is quite marked, doubtless owing to fatigue. Thus the first four experiments in Table III, *viz.*, 29, 30, 31, 32, were characterised by low values for the chlorine concentration; but in the next experiment (*viz.*, 33), where the wet-bulb temperature of the air in the chamber had been raised, there was a distinct rise in the chlorine percentage as well as in the weight of sweat excreted; this was also evident in experiments 34, 35, 36 (Table IV).

The effect of the performance of work on the salt concentration of sweat is a matter which has a distinct practical bearing. In experiment 23 (Table I) the subject was doing work in the first and third sections, but was at rest during the second section. The results show a variation in the chlorine concentration which is almost negligible, and the percentage of potassium only varies very slightly. In other tests where the subject was at rest during each section, experiments 38 and 39, the difference from experiments in which work was performed lay in the progressive rise in chlorine concentration. This

was not evident, however, in experiments 41 and 43 where only slight sweating occurred. It must be noted that the amount of work performed in any of the experiments was never very great, and the rate of work was never more than two-thirds of that for an average working miner. The difficulty of discerning the effect of work on the salt concentration would appear to be in eliminating the other variable factors in the case, the chief of these being, perhaps, the physical condition of the individual.

The absolute percentage of potassium in the sweat did not vary to any marked extent, and was of the same order as that found with previous subjects. The percentage of chlorine calculated as potassium chloride showed, however, a very large variation, the lowest values being found, as might be expected, when the chlorine concentration was high in consequence of fatigue of the sweat-glands. It is obvious that this ratio must vary inversely as the chlorine concentration, provided that the percentage of potassium remains constant. The percentage of chlorine as potassium chloride was much higher, particularly in the second and third sections of an experiment, than was found previously, and is more in agreement with an isolated analysis by Ivon Graham, quoted by Moss (1924), which showed that 40 per cent. of the chloride present in sweat was in the form of potassium chloride. A high value for this ratio is apparently due to individual differences in the composition of sweat and not merely to a personal taste for a diet rich in potassium, since the fact that this ratio was not raised by the subject taking large doses of potassium chloride has been shown in previous experiments (1929).

It was desirable to ascertain if appreciable amounts of inorganic salts other than chloride were present in sweat, particularly during the later stages of a test, and also to obtain more data with reference to the percentage of organic matter. Analyses of the ash of some of the samples of sweat were therefore performed.

Analysis of Sweat Ash.—After evaporating the sweat and washings to dryness, the solid matter was transferred to a platinum boat, and the latter placed in a length of combustion tubing which was fitted into an electric furnace. By this procedure the sweat could be ashed in a stream of oxygen at a temperature of 650° to 700° C. without any loss of chloride.

A typical result is shown in the table below :—

Experiment 34 (c)—

Organic matter	0.086 per cent.	} solid matter 0.368 per cent.
Ash	0.282 ..	
Chlorine (Cl)	0.144 ..	
Sulphate (SO ₄)	0.015 ..	

From Table IV it will be seen that the chlorine in the original sweat and washings was 0.145 per cent., and that the percentage of chlorine calculated as KCl was 17. If the chlorine and sulphate in the ash are each calculated as being 17 per cent. combined with potassium and 83 per cent. with sodium, the percentage of sodium and potassium chloride is 0.250, and sodium and potassium sulphate 0.023—giving a total of 0.273 per cent. This latter figure will be seen to account very nearly for the whole of the ash, viz., 0.282 per cent. The figures for the experiment above are for the third hour of the test, and the loss in weight was low (220 grams); but a considerable amount of sweat was excreted during the first and second periods, viz., 990 grams and 615 grams respectively. Thus in the later stages of a test after considerable sweating, the proportion of organic matter to ash is about 1 : 3, and practically all the inorganic matter is in the form of chloride, since, as shown below, most of the sulphate is formed during the process of ashing.

The change in the proportion of organic matter to ash during the course of an experiment is shown in the table below :—

Experiment No.	Weight lost through skin.	Solid matter.	Ash.	Organic matter.
	grams.	per cent.	per cent.	per cent.
27 (a)	430	0.200	0.108	0.082
(c)	300	0.207	0.166	0.051

The ratio of organic matter to ash was approximately 1 : 1 in the first period of the experiment, while in the third period it had fallen to 1 : 3. This explains the fact that in the previous results the proportion of organic matter to ash was found to be greater in experiments where the total loss in weight was small than where it was large. An estimation of sulphate in the sweat and washings of experiment 27(a) gave a figure of 0.007 per cent., while the ash of the same liquid gave 0.021 per cent. Most of the sulphate in the ash was, therefore, not present in the sweat as such, but probably as organically combined sulphur. Phosphorus determinations were carried out on the sweat and washings of experiment 27(a) and (c) but gave negative results, and any phosphate present could only have amounted to traces. The probability of contamination of sweat in the early stages of a test with the products of broken-down cells from sweat-glands and other sources (mentioned in the previous paper) is strengthened by the figures quoted above for the variation in the proportion of organic matter to ash.

III.—*Air Conditions as determined by Wet-bulb Temperature and Wet Kata Cooling Power.*

Although wet Kata cooling powers are included in some of the experimental results, it is now recognised that, with low readings, the values do not give a true measure of the effect of the air conditions on an individual. The directions now given with a Kata thermometer state that it "does not measure the cooling power of the air on the human body, but enables one to measure conditions which are comfortable and invigorating, and so secure these in rooms." It will be seen from the results that with a wet Kata cooling power of from 4 to 5 the subject was able to perform a moderate amount of work without serious discomfort, *cf.* experiments 26 and 31. After 3 hours in the chamber with the cooling power approaching 2.5 it was apparent that the subject would soon have to stop work or come out of the chamber, as the mouth temperature and resting pulse rate were rising continuously, the latter reaching 140 at the finish of experiment 22. Pulse rate reacts more sensitively than mouth temperature to the condition of the surrounding air, and this will be seen in Tables I to V.

When the subject was at rest, values for wet Kata cooling power could not be obtained in many cases since the wet-bulb temperature was very near to 95° F., and in some cases was closely approaching 96° (*cf.* experiment 43). In experiment 41, the average air temperature in the chamber was 94.6° F. saturated, and only a small rise in mouth temperature occurred after 3 hours, while the pulse rate finally reached 110. The weight lost through the skin was very low and the subject scarcely appeared to be sweating visibly. With a chamber temperature of 95.8° F. saturated, the pulse rate gradually mounted to 124 in 3 hours (experiment 43), although the mouth temperature had only risen to 99.7° F. In one of the earlier experiments (No. 21) at rest, which was continued for 4 hours, the air temperature was 94.5° F. saturated in the final hour, and the pulse rate reached 140 and the mouth temperature 100.6° F., the subject was unable to regulate his body temperature and further prolongation of the experiment would have involved the risk of unpleasant symptoms. McConnell and Houghten (1923) found that in general "very uncomfortable sensations are felt after the pulse rate exceeds 135 pulsations per minute, and the atmospheric condition becomes unbearable when the rate exceeds 160 per minute."

The results of the experiments in Tables I to V indicate that a moderate amount of work (1700 foot-lbs. per minute) can be performed without undue

discomfort with a wet Kata cooling power of about 3, provided that the subject is to some extent acclimatised to the conditions. The value of ascertaining the minimum wet Kata-thermometer reading at which miners can or will accomplish their work has been pointed out by Haldane (1929); for, if we know this, we can infer from the actual Kata-thermometer reading what available reserve of physiological regulating power they possess.

The critical wet-bulb temperature above which a well-acclimatised man cannot stay very long at rest in still air in an experimental chamber without his body-temperature rising abnormally and continuously was estimated by Haldane (6) to be about 93° F. Provided that there is some small air motion, it appears from the results of the experiments that this figure can be increased to 94° or 95° F., depending to a large extent on the physical condition of the subject at the particular time. It must not, however, be concluded that these values are of general application, for, as has been stated above, the temperature of the walls of the chamber in these experiments was slightly below the air temperature. The conditions, therefore, are by no means strictly comparable with those obtaining, say, in a mine roadway where the temperature of the surrounding strata is usually as high as the air temperature.

It will be noticed in Table V that although the wet-bulb temperature in the experiments at rest and at work was higher than that in Tables I to IV, the subject was able to withstand the former conditions quite as well as the latter, and in some cases (in particular, those at rest) actually with more comfort. This is no doubt explained by the fact that the subject had for some months prior to the last group of experiments (Table V) been working underground, whilst previously he had been engaged on surface work. Mouth temperatures up to about 100° F. should not be regarded as abnormal in experiments of this nature, since the normal stimulus to sweating is a slight rise in the blood temperature. Thus in experiments 39 and 41 where the rise in mouth temperature was quite small, the sweat excretion was of a very low order.

Summary.

(1) For a given rise in body temperature sweating is facilitated by the performance of muscular work, when compared with sweating produced by the same rise in body temperature with the subject at rest. It seems probable that the extra stimulus to sweat production is due to some product of muscular metabolism.

(2) The performance of a moderate amount of work appears to be accompanied by little rise in the chlorine concentration of the sweat, while a marked

increase with time is evident when the subject is at rest and the sweating is simply due to the wet-bulb temperature of the surrounding air. A progressive decrease in the proportion of organic matter to ash is observed as sweating continues. Although a small amount of sulphate was found in the sweat during the first period of an experiment, only traces of sulphate were found in the sweat collected during the final stages of a test.

(3) The sweat of the subject of these experiments showed a lower concentration of chlorine and a much higher value for the percentage of chlorine as potassium chloride than was found with the subjects of previous experiments. It is evident from this that not only the chlorine concentration but also the ratio of chlorine to potassium in the sweat varies for different individuals.

(4) A moderate rate of work could be maintained when the surrounding air had a wet Kata cooling power of about 3, the wet-bulb temperature being approximately 90° F. When the subject was at rest in the experimental chamber an air temperature of 95° F. saturated (i.e., the wet Kata cooling power at zero) caused little disturbance to regulation of the body temperature. These figures, however, appear to be dependent to some extent on the acclimatisation of the subject to the experimental conditions, as well as on the walls of the chamber being slightly cooler than the air.

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A Crystalline Antirachitic Substance.

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The production of small quantities of a crystalline substance of high antirachitic activity, by the vacuum distillation of the products of irradiation of ergosterol, was described by Askew *et al.* (1930, *a*). We have now been able, by improving the technique of preparation, to obtain this substance in larger quantity, and in a state of increased purity.

The more detailed study of its behaviour which has now been possible has shown that the intense antirachitic activity is almost certainly inherent in the pure compound, and not due to a contaminant.

Owing to the exceptional difficulty of purification in the sterol group, we cannot yet be certain that this substance is a single compound, but we think the provisional adoption of a name is justified, for the sake of convenience, and suggest "Calciferol" in view of the high antirachitic activity of the substance.

The following account is divided into four sections, describing, respectively, the preparation of the crystalline substance, its properties, its probable degree of purity, and its relation to normal vitamin D.

PREPARATION OF THE CRYSTALLINE PRODUCT.

The method of preparation now used is a modification of that described by Askew *et al.* (1930, *a*). The following account describes chiefly the changes made in the process, and for fuller details reference should be made to the former paper.

The ergosterol used in this work was supplied on special terms by Messrs. Boots, Messrs. British Drug Houses, and Messrs. Burroughs Wellcome, to whom we wish to make acknowledgment. It had been recrystallised from a mixture of alcohol and benzene, and the only impurity present in significant quantity was about 5 per cent. of α -dihydroergosterol, which could not affect

the results obtained, since it is practically unaffected by irradiation, and is removed by digitonin (Callow, 1931).

Irradiation of Ergosterol.

A 0.3 per cent. solution of ergosterol in pure, dry, air-free ethyl ether was allowed to flow through a water-cooled spiral of silica tubing of 4 mm. bore, closely surrounding a vacuum-type mercury vapour lamp. No light filter was used, and the rate of flow was kept between 20 and 30 c.c. per minute, so that between 75 and 60 per cent. of the ergosterol was transformed.

Removal of Unchanged Ergosterol.

The irradiated solution was evaporated to dryness under reduced pressure, and at a temperature of 25° to 35° C. The residue was then treated with 90 per cent. alcohol, or, in a few cases light petroleum, at about 0° C., and the solution filtered from undissolved ergosterol. In the first three preparations the residual ergosterol was then removed from the alcoholic extract by precipitation with digitonin, evaporation of the filtrate to dryness, and extraction of the residue with ether. The ethereal solution was then evaporated to dryness before distillation. In the later series the filtrate from the undissolved ergosterol was evaporated and distilled directly. The fractions of distillate then contained small amounts of ergosterol, which were removed by precipitation with digitonin, as described in the section on crystallisation.

Distillation.

The distillation and fractional condensation were carried out in the high-vacuum still of 55 mm. bore described previously, (Askew *et al.*, 1930, a). The temperature at the boiler end was kept at 150° C. for 2 hours, and the distillate condensed as before, at temperatures ranging from 140° C. to less than 100° C. It was found that the distillate could be divided into two main fractions, of which that condensing at 125° to 140° C. would not crystallise, while the portions condensing below 125° C. crystallised well.

The redistillation previously carried out was found to be unnecessary. About 2 g. of irradiation product was distilled at a time, and of this about 26 per cent. was usually left undistilled, and about 37 per cent. was collected in each of the two fractions.

Crystallisation.

The more volatile fraction of the distillate was collected by removing the lining tubes from the still, and washing the product into weighed flasks with

ether. The ether was evaporated, at first in a current of nitrogen, and finally under reduced pressure. The solutions occasionally crystallised spontaneously during this evaporation. The residue was dissolved in 100 parts of warm methyl alcohol, and 16 parts of water were added, forming a turbid solution. On cooling, crystals separated, sometimes only after seeding, and were collected after standing for 24 hours at 4° C. Some variation in the proportion of solvent to solute was found possible without apparent loss, and on some occasions acetone or ethyl alcohol was used instead of methyl alcohol. The average yield of crude crystals from a series of 21 distillations was 46 per cent. of the weight of the more volatile fraction of the distillate, or 17 per cent. of the original resin used for distillation. The highest yield observed was 34 per cent. of the original resin. If all ergosterol had not been removed before distillation, the crude crystals were treated with excess of digitonin in boiling 90 per cent. alcohol. After standing in the cold, the precipitated ergosterol digitonide was removed by filtration, and the filtrate was warmed, diluted with water until cloudy, seeded and allowed to crystallise at 0° C. The product was then crystallised from acetone to remove traces of digitonin. The recrystallisation was repeated one or more times, usually from methyl alcohol. In some cases the distillation products were yellow in colour, and then the product was boiled with charcoal to reduce the coloration.

The following is a typical example of the procedure. The distillate (0.812 g.) was recrystallised from aqueous methyl alcohol, and yielded 0.309 g. of crystalline material. This was dissolved in 90 per cent. alcohol (15 c.c.), and treated with 0.3 g. of digitonin in 15 c.c. of 90 per cent. alcohol. The filtrate from the digitonide yielded 0.190 g. of material, which, recrystallised from 2 c.c. of methyl alcohol, yielded 0.119 g., m.p. 119–121.5°, $[\alpha]_{5461}^{20} + 256^\circ$ in alcohol. A further recrystallisation from 60 per cent. ethyl alcohol yielded 0.075 g., m.p. 123–125°, $[\alpha]_{5461}^{20} + 258^\circ$.

Visible, oily impurities are readily removed by recrystallisation from methyl alcohol, but complete purification is not effected quickly, or without considerable loss, from any of the solvents used.

Precautions against Oxidation.

It appears necessary to take careful precautions against oxidation during irradiation, and throughout the other stages preliminary to distillation. For this purpose commercial 99.5 per cent. nitrogen was purified by passage through vessels containing copper wire immersed in an aqueous solution of

ammonia and ammonium chloride, and then through a washing and drying train.

This purified nitrogen was used in preparing the solution of ergosterol in oxygen-free ether, and, in all manipulations prior to distillation, whenever evacuation was unsuitable. We think it probable that the purification of the nitrogen has been an important factor in the increased ease of crystallisation which has been obtained since the work published in 1930.

PROPERTIES OF CALCIFEROL.

Methods of Quantitative Measurement.

(a) The antirachitic activity was measured by the radiographic technique described previously by Bourdillon *et al.* (1929). The results are expressed in terms of Medical Research Council units per milligram. Thus, the statement that calciferol has an activity of 20,000 means that each milligram of calciferol contains 20,000 Medical Research Council units. The same result could be expressed by saying that 1 mg. of calciferol has twice the antirachitic activity of 1 mg. of the irradiated ergosterol used in preparation of the M.R.C. standard solution, as now issued from this Institute.

It may be pointed out that the results now found for calciferol are almost identical with those recorded in the paper on distillation by Askew *et al.* (1930, *a*), when correction is made for the difference in units employed, and for the fact that the former workers used a somewhat stronger standard solution.

A detailed study of the errors in our antirachitic tests has now been made, and will be published later. We find that the frequency of errors of different magnitudes closely follows the distribution given by the "normal curve." It is therefore correct to say that errors of, or exceeding, twice the standard deviation of the results for single tests will occur once in 22 tests. We have taken this frequency as a practical limit, and found that the corresponding error for tests depending on 10 pairs of rats (half on standard solution and half on the test solution) is +40 or -29 per cent. The majority of tests described in this paper were based on more than 10 pairs of rats, and the lowest number used was 8 pairs. We can therefore say that measurements of antirachitic activity are probably correct within +40 or -29 per cent. It may be remarked that the variation between results for the 18 tests shown in Table I is well within this range.

(b) *Measurement of Absorption Spectra.*—The absorption spectra were measured in alcoholic solution by a photographic method, using a hydrogen

tube as source of light, a Hilger logarithmic sector, and a microphotometer, as described in a previous paper (Bourdillon, *et al.*, 1930). The extinction coefficients plotted in the diagrams in this paper are calculated by the formula

$$\epsilon = (\log_{10} I_0 - \log_{10} I_1 - K)/Cd,$$

where I_0 and I_1 are the intensities of light before and after traversing the solution, C is the concentration in grams per litre, and d is the thickness of the layer of solution in centimetres. K is the figure representing the absorption of the silica cell when filled with solvent only. This figure is subtracted from the total absorption, in order to give the absorption due to the dissolved substances only.

(c) *Polarimetry*.—The specific rotations of the specimens (shown in Table I) were measured using the mercury green line (5461 Å.U.), in 99 per cent. ethyl alcohol, generally at a concentration of about 0.2 per cent., in a 4 dm. tube, with an instrument reading to 0.01°. The expected error in specific rotation, due to all causes, is estimated to be within 2°. The measurements plotted in figs. 3 and 4 were made on dilute solutions of concentration 0.01 per cent., and have an appreciably larger error.

(d) *Melting Points*.—The melting points were determined in sealed capillary tubes heated slowly, and are uncorrected.

Physical Properties.

The most reliable values that we have obtained for the properties that we have measured are as follows :—

m.p. 123–125°.

$[\alpha]_{5461}^{20} + 260^\circ$ in alcohol (concentration 0.2 per cent.).

Extinction coefficients—

λ	290 $m\mu$	282 $m\mu$	271 $m\mu$	247 $m\mu$
ϵ	15.3	25.6	33.7	19.3

The absorption spectrum is shown in fig. 1, together with that of purified ergosterol. It is interesting to note that a curve of this type, with maximum at or near 270 $m\mu$, has been attributed to vitamin D by various workers.

The compound is very soluble in most organic solvents. It may be recrystallised from about 10 parts of methyl alcohol or acetone, or from aqueous ethyl alcohol. It forms sheaves of small needles from methyl alcohol, or long, flat needles from acetone or aqueous ethyl alcohol.

No. of sample.	Melting point.	[α] _D ²⁵	Antirachitic activity units per mg.	Extinction coefficients.				Remarks.
				390 mμ.	283 mμ.	271 mμ.	247 mμ.	
A. Normal Preparations.								
5a	112-123	0	15,300	17.5	27.8	35.2	22.0	—
5b	120-123	—	20,000	17.9	28.0	34.1	20.7	—
6a	104-115	—	18,600	—	—	—	—	—
6b	116-122	—	—	15.0	26.9	32.9	20.5	—
6c	131.6-123	+236	20,100	14.0	24.5	31.0	18.8	—
7a	108-118	+232	—	—	—	—	—	—
8a	117.6-122	+249	—	—	—	—	—	—
8b	123-125	+247	22,400	15.1	25.9	33.7	21.5	—
8c	123-124.5	+246	22,500	16.0	26.8	32.6	21.6	—
8d	—	—	—	—	—	—	—	—
10c	116-123	+243	—	16.9	27.1	32.8	21.2	—
10d	120-123	+250	18,200	—	—	—	—	—
11c	119-121.5	+256	—	14.8	25.5	30.5	19.7	—
11d	123-125	+258	22,000	16.3	26.4	33.1	21.4	—
12c	121-124	+260	17,200	18.0	25.0	32.7	20.4	—
14c	121-124.5	+254	—	15.4	25.3	31.9	19.7	—
14e	—	+253	—	—	—	—	—	—
B. Preparations that have received special treatment.								
8d.L.44d	—	+243	21,600	15.9	27.2	33.8	22.1	Tested after keeping for 44 days in dry air.
6c.L.56d	—	—	22,000	16.0	26.9	32.2	22.4	Tested after 56 days in <i>vacuo</i> .
11a2b	115-119	+240	20,000	15.0	26.6	31.1	20.9	Obtained by hydrolysis of the oxalate.
D81c	119-123.5	+234	21,300	16.3	26.2	32.8	21.2	Obtained from 10d by two further distillations and then recrystallization.
10d.x3	104-111	+211	17,600	14.8	22.6	28.8	22.6	Made from a specimen (10d) that had been radiated by long waves until 2/3 was destroyed.
7cc5	113-117	—	17,000	14.2	23.2	28.4	19.6	Recovered from an oil formed in an attempt to produce an acetate.
14d.M	—	+229	21,000	15.4	25.9	31.1	21.3	Two fractions obtained from 14d by shaking with methyl alcohol and hexane, and recovering from the two layers separately.
14d.H	—	+253	18,900	15.6	26.6	32.2	22.9	
7f	120-124	+246	25,000	14.2	24.5	32.0	19.5	After treatment with "norite" charcoal in methyl alcohol.

Table I shows the melting point, specific rotation, and antirachitic activity, as far as these have been measured, for most of the samples of crystals that we have obtained. The numbers in the first column each refer to a sample made by a complete repetition of the process of production from exopteron. The small letters *a*, *b*, *c*, *d*, *e*, immediately following the numbers denote the number of times the specimen had been recrystallized. Thus, "a" denotes crude and usually oily crystals. If these have been recrystallized once, the product has the letter "b," if twice, the letter "c," and so on.

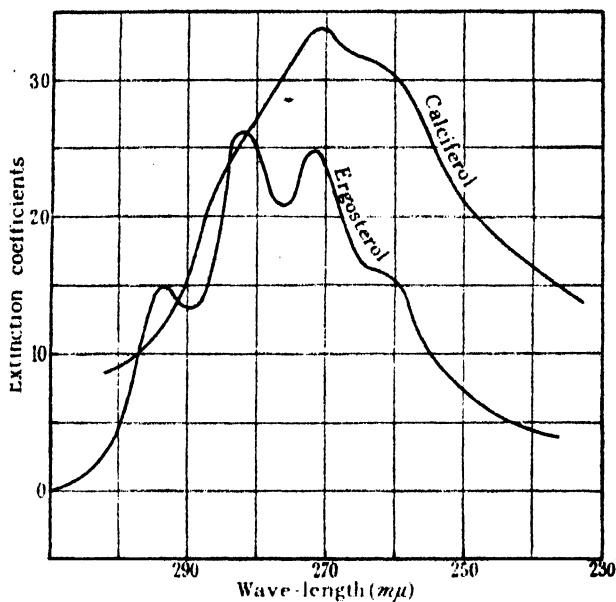


FIG. 1.—Absorption Spectra of Calciferol and Ergosterol.

Antirachitic Activity.

The mean antirachitic activity of the different samples is 20,000 units per milligram, and thus is approximately equal to the best values that we have been able to obtain for the resinous products of irradiation of ergosterol, after removal of unchanged ergosterol.

Stability.

The purified substance appears surprisingly stable when stored, either *in vacuo*, or in dry air, for 1 or 2 months. 2.03 mg. of sample 6c kept *in vacuo* for 56 days showed no detectable change in absorption spectrum, or in antirachitic activity (No. 6c.k56d, Table I). This stability is in striking contrast to the behaviour of the crude products of irradiation of ergosterol as observed by Windaus (1930), and by ourselves, and also in contrast to the behaviour of the impure crystals recorded previously by Askew *et al.* (1930, *a*). Another specimen (8d) was kept for 44 days in air, in a weighed vessel, over phosphorus pentoxide in a desiccator shielded from light. Weighings at intervals of a few days showed no change in weight, and at the end of the period no certain change could be detected in the optical rotation, absorption, or antirachitic activity (Table I, No. 8d.k.44d). The results indicate that the

stability of calciferol is of the same order as that of ergosterol itself, and that it may be possible to use this substance as an absolute standard of antirachitic activity.

Elementary Analysis and Molecular Weight.

We are indebted to Mr. H. P. Marks for the micro-analysis of one of the earlier samples (No. 6c). The mean of three determinations gave, C, 84.9 per cent. ; H, 11.1 per cent. The theoretical values for $C_{27}H_{42}O$ are C, 84.7 per cent. ; H, 11.1 per cent. The molecular weight as found by the depression of the freezing point of camphor (Rast's method) was 370, in fair agreement with the calculated value for $C_{27}H_{42}O$ of 382. These results indicate that the substance is probably an isomeride of ergosterol.

Colour Reactions.

The following colour reactions of the crystalline substance are clear and definite, in contrast to those of the crude irradiation products.

Concentrated sulphuric acid : bright red. Salkowski : acid layer bright red. Liebermann-Burchard : yellow $\rightarrow$ red $\rightarrow$ intense emerald green. Bromine (Tortelli-Jaffé), blue-green. Iodine in acetic acid (Rosenheim and Callow, 1931), brilliant green without selective absorption, in contrast to ergosterol, which gives a bluish-green, developing more slowly, with absorption bands at 550 and 600 $m\mu$. Antimony trichloride : bright yellow. Mercuric acetate in nitric acid (Rosenheim and Callow, 1931) : chloroform layer evanescent pink changing to deep yellow, then yellow in both layers. Trichloroacetic acid : pink, changing rapidly to orange, then yellow-brown. The pink colorations with the last two reagents suggest the presence of a $\Delta^{1,2}$ (or $\Delta^{1,23}$) linkage (Rosenheim, 1929).

Chemical Reactions.

The substance does not form an insoluble digitonide. Attempts to prepare an oxime or a semicarbazone by the usual methods have failed. On the other hand, attempts at esterification have been partially successful, and afford strong evidence that the compound contains an alcoholic hydroxyl group.

Esterification.

(a) *Preparation and Hydrolysis of an Oxalate.*—82 mg. of calciferol were dissolved in a mixture of ether (5 c.c.) and pyridine (2 c.c.). A solution of oxalyl chloride in ether (about 1 c.c. of 20 per cent.) was added drop by drop, with constant shaking and stirring, until the mixture acquired a yellow colour,

which persisted after boiling for a few minutes. The mixture was poured into water. The ethereal layer was separated, washed with dilute acetic acid and water, dried over sodium sulphate, allowed to stand with charcoal, and evaporated to dryness. The resinous residue (83 mg.) was dissolved in ethyl acetate and precipitated by the addition of methyl alcohol. The precipitate was an amorphous powder (weight 30 mg.) which could not be obtained crystalline from a number of solvents which were tried.

On warming with very dilute aqueous alcoholic sodium hydroxide, hydrolysis occurred, and the original crystalline material separated on dilution with water. After one recrystallisation from aqueous methyl alcohol, this showed a melting point, rotation, absorption spectrum, and antirachitic activity differing only slightly from those of the original specimen of calciferol, thus proving their identity (*cp.* Table I, Nos. 11*d* and 11*ox.b*). In the aqueous solution of the products of hydrolysis of the ester, the presence of oxalic acid was easily demonstrated.

(*b*) *Preparation and Hydrolysis of an Acetate*.—40 mg. of calciferol were boiled under reflux for 5 minutes with 1 c.c. of acetic anhydride. After pouring the mixture into water, and keeping at 4° for 2 days, the oil which separated was washed with water. No crystalline material could be isolated from a number of solvents tried. The oil was sparingly soluble in methyl alcohol, and was washed with this solvent, leaving a residue which was boiled with 2 c.c. of 20 per cent. alcoholic sodium hydroxide, in which it dissolved slowly. When the mixture was carefully diluted with water, an oil separated which soon solidified, m.p. 113–117°. Recrystallised from aqueous methyl alcohol, needles were obtained, m.p. 113–117°. A mixture with the original unesterified material melted at 117.5–121°. The absorption spectrum and antirachitic activity proved to be only a little lower than those of the original substance, and so confirmed the identity of the recovered product with calciferol (Table I, No. 7*b*, 7*ac.b*). Attempts to obtain other esters have so far only led to ill-defined oily products.

Toxic Effects of Large Doses.

It is well known that large doses of irradiated ergosterol are toxic, and cause loss of weight and death, usually with the deposition of calcium in the heart or aorta, and kidneys. Windaus (1930) and Holtz and Schreiber (1930) have shown that it is possible to obtain products from ergosterol which show high toxicity with little or no antirachitic action, and conclude that vitamin D itself is not necessarily toxic even in large doses. Others, such as Harris and

Moore (1928), and Harris and Innes (1931), claim that vitamin D is itself toxic in large doses. We have therefore performed a few tests of toxicity on calciferol, although we have not been able to make full determinations of an accuracy comparable with that of other workers.

Adult female rats about 14 months old and 220 gm. in weight, were fed on a mixed diet containing normal amounts of calcium and phosphorus, and given daily doses of the desired amount of calciferol dissolved in each case in 0.1 c.c. of olive oil. The doses were given by capillary pipettes into the animals' mouths.

The sample of calciferol used was one of the purest specimens obtained (13e) and was given in three different doses. Two rats received 0.2 mg. daily for 22 days, and showed negligible losses of weight of 6 and 13 g. respectively, but no other perceptible effects. Two rats received 1 mg. per day, and showed large falls in weight of 50 and 51 g. respectively in 28 days. They were then killed by coal gas, and the heart and kidneys examined. Radiographs of the excised organs showed no calcium deposits. Two rats received doses of 2 mg. daily for 17 days. Both died on the 17th day, after a loss in weight of 85 g. and 68 g. respectively. Post-mortem radiographs showed considerable calcification of the heart, and of the wall of the stomach, but no visible deposits in the kidneys. Histological examination showed extensive deposition of calcium in the heart (muscle and A.V. junction), in the stomach (muscle and mucosa), and in the kidneys (arterioles and straight and convoluted tubules) in these two specimens. For comparison, tests were made with doses of 2 mg. and 0.2 mg. of a mixed irradiation product of ergosterol, from which the greater part of the 33 per cent. of unchanged ergosterol had been removed by treatment with cold alcohol. The radiation had been carried out in the spiral, using unfiltered light as described earlier in this paper. The results were closely similar to those obtained with calciferol.

Owing to the small number of tests performed, these results must be interpreted with caution. They appear, however, to show that calciferol is of approximately equal toxicity to a normal irradiation product. It should nevertheless be pointed out that the discovery by Windaus (1930) of the production of highly toxic substances by heating irradiated ergosterol makes it conceivable that the apparent toxicity of calciferol is due, not to the substance itself, but to traces of a toxic contaminant.

Effect of Ultra-violet Radiation.

Owing to their considerable interest, a series of tests was made showing the rate of destruction of calciferol by ultra-violet irradiation. In these tests the crystals were dissolved in absolute alcohol (air-free) to a concentration of 0.01 per cent., or 0.0025 per cent., and irradiated in a silica cell with plane walls 1 cm. apart, by a mercury vapour lamp (atmospheric pressure, K.B.B. type, 2.5 amps. 120 volts across the arc). A light filter was always used, either (A) a 1 per cent. solution of xylene in alcohol, excluding wave-lengths shorter than 275 $m\mu$, and transmitting freely wave-lengths longer than 280 $m\mu$, which are referred to in this paper as "long waves," or (B) two separate cells containing layers 2.5 cm. thick of chlorine gas, and of bromine vapour respectively; these transmitted chiefly "inactive" wave-lengths longer than 600 $m\mu$, and "active" wave-lengths between 210 $m\mu$ and 280 $m\mu$. Although the layers of gas were too thin to exclude wholly the wave-lengths between 280 $m\mu$ and 600 $m\mu$, the greater part of these were absorbed, and the transmitted radiation is here referred to as "short waves." The distance from lamp to solution was 7.5 cm. when filter A was used, and 11 cm. with filter B. Before, and during, irradiation the solutions were carefully protected by purified nitrogen from contact with air.

In the first test, solutions of calciferol were irradiated in 0.0025 per cent. solution and photographed (for absorption measurements) at intervals, without removal from the cell. The resulting changes in absorption are shown in fig. 2 for the two wave-lengths of 290 $m\mu$ and 271 $m\mu$. It was found that irradiation by long waves produced a continuous, rapid fall in absorption at all wave-lengths. Irradiation by short waves also produced a fall in absorption at 271 $m\mu$, but this fall was irregular, suggesting the formation of an intermediate product, which was itself more slowly converted on further irradiation to a product of negligible absorption. This was fully confirmed on examining the data for the changes in absorption at other wave-lengths which were caused on irradiation by short waves. At 247 $m\mu$ the fall in absorption was continuous. At 271 $m\mu$ the curve showed signs of a plateau. At 282 $m\mu$ the plateau was well marked, and at 290 $m\mu$ the initial fall was replaced by a rise as shown in fig. 2.

These changes are further illustrated by the curves shown in fig. 6, which show that short waves form an intermediate product with maximal absorption at a wave-length slightly exceeding 282 $m\mu$. This product is itself destroyed much more slowly than the original calciferol. Fig. 5 shows the corresponding

absorption spectra for the products of irradiation by long waves, and shows a regular decrease in absorption at all the longer wave-lengths. It is note-

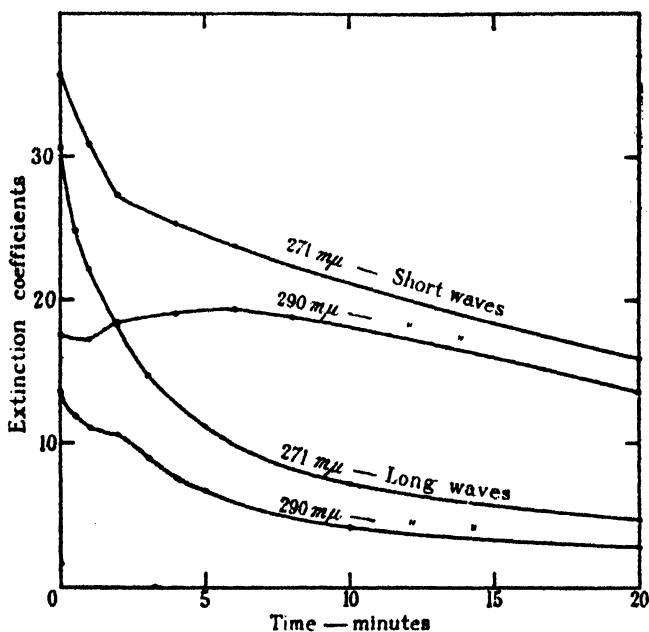


FIG. 2.—Changes in absorption at wave-lengths 271 and 290 $m\mu$ produced in calciferol by long waves and short waves.

worthy that the regular fall produced by long waves, and the intermediate production by short waves of a substance with maximal absorption at or near 280 $m\mu$ are reminiscent of the changes produced on re-irradiation of ordinary undistilled products of ergosterol which had been originally exposed to long waves only (Askew *et al.*, 1930, b).

A second test was carried out in which alcoholic solutions of the calciferol of concentration 0.01 per cent. were irradiated, and then used for measurements of absorption, rotation and antirachitic activity. In this series a separate portion of solution was used for each period of irradiation.

The results of irradiation by long waves are shown in figs. 3 and 5. In fig. 3 the measurements of absorption at 271 $m\mu$ are shown as dots on the continuous curve. The measurements of rotation are shown as crosses, and the measurements of antirachitic activity as circles. All three are plotted to corresponding scales. The small arrows represent the upper and lower limits of probable error for the antirachitic tests. Owing to the small concentration of the solution the probable error of the polarimetric measurements is appreciable

even in the higher readings, and at the lowest point might even reach 15

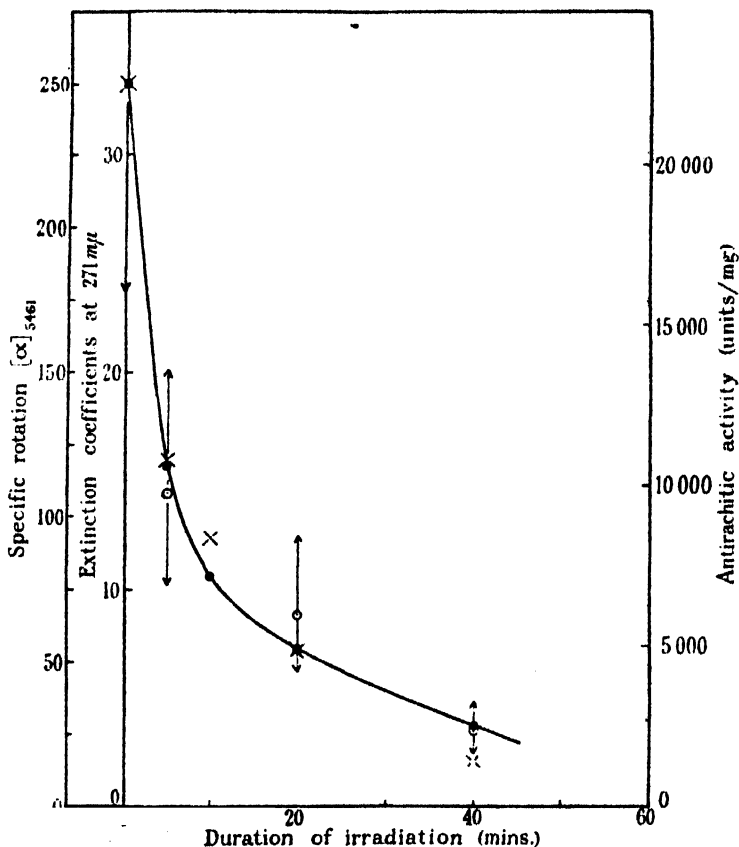


FIG. 3.—Changes in properties of calciferol on irradiation by long waves. ● extinction coefficients; × $[\alpha]_{5461}$; ⊙ antirachitic activity. Arrows show limits of maximum errors in antirachitic activity expected once in 22 times.

These results show a striking agreement in rate of destruction of the three properties of absorption, rotation, and antirachitic activity. This agreement shows that, either all three properties are due, mainly, if not entirely, to one single substance, or that, if the different properties are due to two or more substances, these substances are destroyed at similar rates. It thus affords strong presumptive evidence that the antirachitic activity of these crystals is an inherent property of the crystals, and is not due to traces of an active contaminant. Study of the reaction velocities shows, however, that the values shown in fig. 3 do not fit any simple reaction, unless it is assumed that there

is a small quantity of a second substance showing absorption and antirachitic activity present in the later stages, owing either to production during radiation

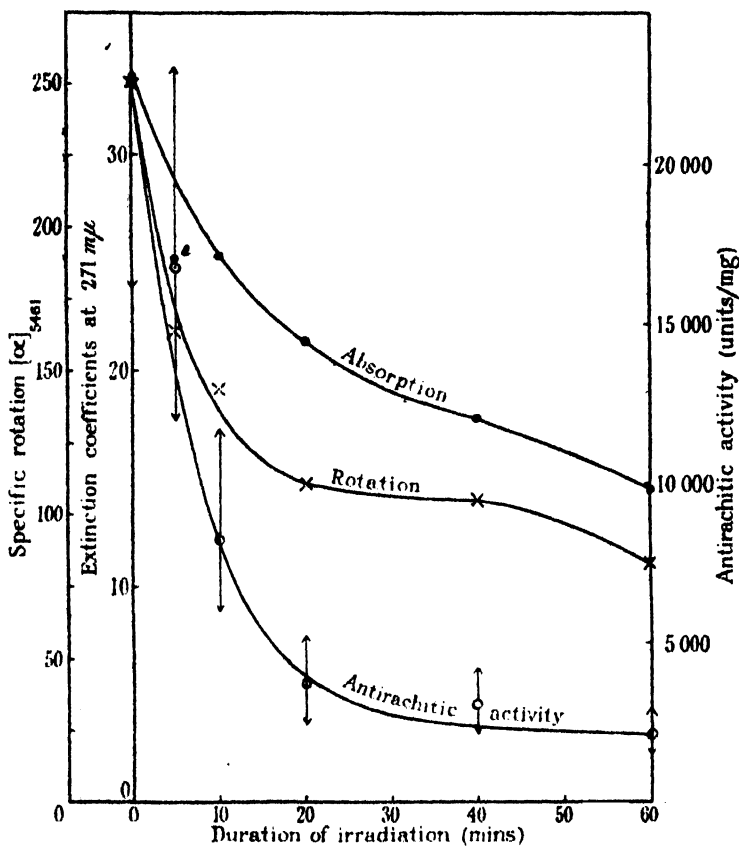


FIG. 4.—Changes in properties of calciferol on irradiation by short waves.

of such a substance, or to the original presence of a small quantity of it in the crystals.

The results for irradiation by short waves are shown in figs. 4 and 6, and are widely different from the above. Fig. 6 confirms the evidence given previously for the formation of an intermediate product showing maximal absorption near 280 mμ, and fig. 4 shows that this product probably has no antirachitic activity, but probably has dextrorotation.

It should be mentioned that the results shown in fig. 4 alone could be explained by the assumption that the crystals contained two substances of different properties. This hypothesis, however, could not in itself explain the rise in absorption at 290 mμ shown in fig. 2, since that necessitates the formation

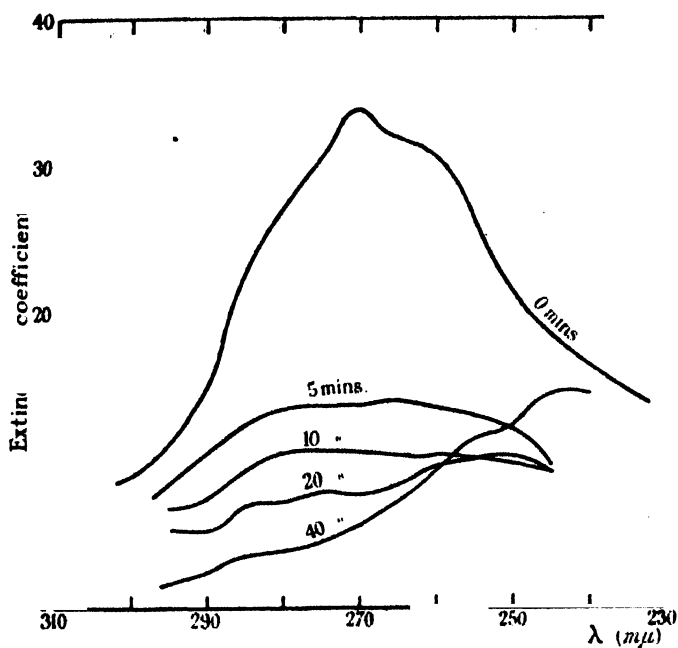


FIG. 5.—Absorption spectra of products of irradiation of calciferol by long waves.

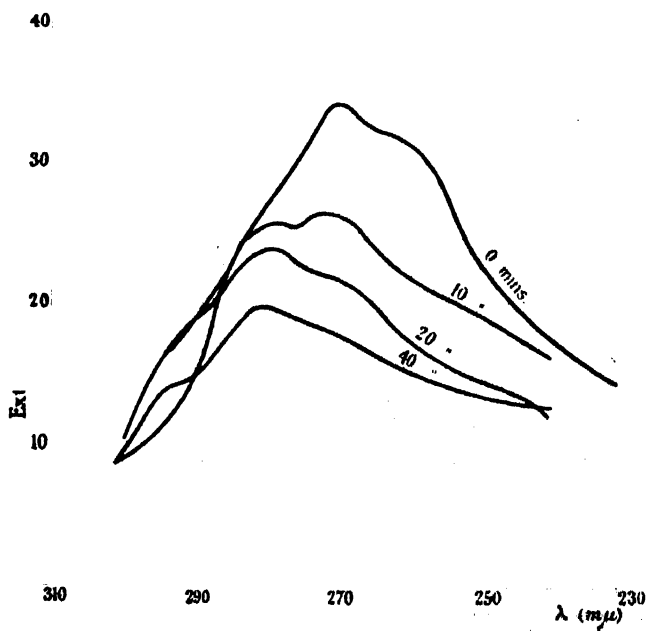


FIG. 6.—Absorption spectra of products of irradiation of calciferol by short waves.

of a new absorbing substance. Accordingly, the results of irradiation by short waves afford evidence neither for nor against the crystals being a single substance.

3. A third test was carried out in an attempt to separate partially one of the two compounds of which the crystals might possibly consist.

It is known that exposure to ultra-violet radiation is a very efficient means of removing ergosterol from cholesterol, and it was thought that, if the crystals did consist of two or more different substances, irradiation would probably exert a preferential destructive action on one of them.

(a) 100 mg. of the calciferol crystals (sample 10d), of $[\alpha]_{5461}^{20} + 250^\circ$, were exposed in 0.01 per cent. solution in ethyl alcohol in a closed silica cell for 3 minutes to long-wave radiation from a mercury arc at 7.5 cm. distance. The specific rotation fell from $+250^\circ$ to $+85^\circ$. On the addition of water a slow deposition of oil and crystals occurred. The latter were recrystallised from aqueous methyl alcohol, and then from acetone, giving 5 mg. of a product of m.p. 104–111°, $[\alpha]_{5461}^{20} + 211^\circ$ and antirachitic activity 17,600 (9 pairs of rats). Although the sample was too small to purify fully, it clearly showed a relation between rotation and antirachitic activity similar to that observed in the original crystals. Hence, if the original crystals did owe their rotation and antirachitic activity to two separate compounds, the latter were either destroyed at approximately equal rates, or else crystallised in unchanged proportions from a mixture containing different relative amounts from those usually present when such crystals are prepared.

EVIDENCE AS TO PURITY OF CRYSTALS.

Unfortunately, the occurrence of solid solutions and molecular compounds is particularly common among the sterols. The impossibility of freeing cholesterol from ergosterol or ergosterol from α -dihydroergosterol by recrystallisation, and the example of " γ -dihydroergosterol," which was shown by Lettré (1930) to be a molecular compound of α -dihydroergosterol and epidihydroergosterol, illustrate the impossibility of assuming that a repeatedly recrystallised product is either pure or simple. The substance we now describe affords a similar problem of especial interest owing to the importance of deciding whether the high antirachitic activity is due to an inherent property of a single crystalline compound, or due to contamination of an inactive crystalline compound with small quantities of a vitamin of an intense activity, exceeding that of any preparation yet made.

We have paid special attention to this question, and have obtained the following evidence bearing on it.

1. *Constancy of Properties*.—The crystals show physical properties of a degree of constancy, as shown in Table I, that would be expected in a single compound, contaminated with small quantities of impurities. Thus they have been prepared on 15 different occasions, and have always shown similar properties, which have approached to a not quite constant limit as the purification proceeded.

2. The evidence afforded by the results of irradiation of the crystals has been given above, and certainly provides support for the view that the crystals are inherently antirachitic, and do not contain large amounts of impurity.

3. *The Effects of Heat and Distillation*.—As mentioned in the paper on distillation (Askew *et al.*, 1930, *a*) our process of distillation and fractional condensation caused considerable destruction of vitamin in ordinary mixtures, and also caused partial separation in mixtures of cholesterol and ergosterol. We therefore applied it twice in succession to a specimen of the crystals, with a view to effecting a partial separation of two constituents, if present.

205 mg. of sample 10*d* of calciferol were distilled at 150° for 2 hours in a still of 36 mm. bore. The vapours condensed partly as resinous droplets, and partly as crystals. 175 mg. condensed between 122° and 95° C., and 75 mg. below 90° C., leaving a residue of 4 mg. undistilled. The less volatile fraction showed $[\alpha]_{5461}^{20} + 154^\circ$, indicating that considerable decomposition had occurred.

170 mg. of this fraction were redistilled for 45 minutes at 150° C. leaving a residue of 48 mg. ($[\alpha]_{5461}^{20} + 54^\circ$), a fraction of 96 mg. condensing at 97–123° C., and showing $[\alpha]_{5461}^{20} + 172^\circ$, and a more volatile fraction showing $[\alpha]_{5461}^{20} + 79^\circ$. The total decomposition was thus considerable. However, the less volatile fraction readily crystallised, giving a product that on recrystallisation from acetone, showed a melting point of 119–122·5°, $[\alpha]_{5461}^{20} + 234^\circ$, and an antirachitic activity of 21,300 units per milligram.

There was thus no appreciable change in antirachitic activity, and no sign of the preferential destruction of one constituent of a mixture.

4. *Partition between Solvents*.—A further test was made in which a sample of calciferol was shaken with two immiscible solvents, namely, hexane and methyl alcohol, in order to effect any separation that might be possible. The two liquids were then separated and evaporated, and the residue examined without further recrystallisation. The data obtained are given in Table I (Nos. 14*d.M* and 14*d.H*), and show no evidence of separation, although the rotation of the

methyl alcohol product is lowered, possibly owing to impurities from the solvent.

As a result of all these tests, we think it probable that the crystals are composed of one substance showing high dextrorotation and antirachitic activity, mixed with a small quantity (perhaps 5 to 10 per cent.) of a second product of lower or opposite rotation. Further, that if this second product also has antirachitic activity, it is not intensely active, and is only responsible for a small part of the activity of the whole crystals.

RELATION OF CALCIFEROL TO "VITAMIN D."

We have no conclusive proof as to the relation of this crystalline compound to the antirachitic substance, or substances, normally produced by the irradiation of ergosterol. We have, however, observed certain facts bearing on this question as follows.

1. The average antirachitic activity of a preparation made by irradiating ergosterol until 30 to 50 per cent. is transformed, and then removing the unchanged ergosterol, is of the same order as the activity of these crystals. On the other hand, the rotation is wholly different, the products being weakly dextro- or laevo-rotatory according to the exact condition of preparation (*cf.* also Windaus, 1930). The crystalline substance cannot therefore be the sole active substance present in a normal irradiation product, since it would have to be present in an amount sufficient to cause a high dextrorotation, in order to produce enough antirachitic activity.

It can, therefore, be stated definitely that if the crystals are essentially a single antirachitic compound (with only unimportant impurities), there must exist not less than two substances possessing intense antirachitic activity.

2. During the process of distillation two extensive changes occur in the properties of the mixture (as measured by considering the sum of all the fractions distilled and the undistilled residue). First, there is a large destruction of antirachitic activity (*cf.* Askew *et al.*, 1930, *a*). Secondly, there is a large increase in dextrorotation, due, either to the destruction of a laevorotatory substance, or to the formation of a dextrorotatory one.

We have no direct evidence to show whether calciferol is a new compound formed by heat, or a normal product of irradiation present in the original resin.

3. The purified calciferol is much more stable *in vacuo*, and in dry air, than is the vitamin contained in normal irradiation products. It is, however, more rapidly destroyed by long waves.

We could infer that the increased stability of calciferol in air was due to removal of some pro-oxidant present in ordinary irradiated mixtures, and similarly that the increased sensitivity of calciferol to long-wave irradiation was due to removal of substances of similar absorption that could exercise a screening effect. An alternative hypothesis is that calciferol is essentially of different stability from the normal vitamin. We think the latter view the more probable.

4. The antirachitic activity of material in the mother liquors from which the crude crystals have been deposited, is often equal to, or even apparently slightly greater than, that of the crystals, although the dextrorotation of the material in the mother liquors is much less than that of the crystals.

This again shows that, if the crystals are a nearly pure antirachitic substance, there must be a second antirachitic substance of laevo- or low dextro-rotation, present in the mother liquors.

Further deductions can only be speculative until more evidence is available. All that can yet be stated is that a large number of facts could be explained by the supposition that the irradiation of ergosterol by long waves produces simultaneously two isomeric compounds each of high antirachitic activity, one showing dextrorotation, and one laevorotation. Such compounds would almost certainly not be enantiomorphic, and would probably show minor differences in stability, in sensitivity to radiation, in the products of their further irradiation, and in their absorption spectra, such as we have observed in comparing the calciferol with the normal early irradiation products produced by long waves. However, this suggestion is merely a guess at one simple theory that would explain our observations.

The suggestion that calciferol is a new product formed by heat from part of the original irradiation product must be considered as one of many possible alternatives.

Since this paper was written we have had the privilege of a discussion with Drs. A. van Wijk, E. H. Reerink and J. van Niekerk, and were interested to learn that the assumption of two antirachitic compounds could be reconciled with their own observations on the product of long wave irradiation of ergosterol.

Summary.

A crystalline antirachitic substance has been obtained by distillation of irradiated ergosterol in a high vacuum.

A total quantity of several grams of this substance has now been produced, as compared with the few milligrams described in a previous paper.

Attempts to detect a possible contaminant of high antirachitic activity have all failed, and it is believed that the observed activity is an inherent property of the substance.

The name "calciferol" is provisionally suggested, pending further elucidation of the structure of this substance.

The substance shows a high antirachitic activity of 20,000 M.R.C. units per milligram, a melting point of 123–125° C., and a high dextrorotation $[\alpha]_{D40}^{20} + 260^\circ$.

It appears to contain an alcoholic hydroxyl group, and to be an isomeride of ergosterol.

It shows unexpected stability, being almost unchanged after keeping for 6 weeks *in vacuo*, or even in dry air at room temperature.

Its relation to "vitamin D" is discussed, and the conclusion is reached that there are probably not less than two compounds each possessing intense antirachitic activity. It is uncertain whether calciferol is normally present in irradiated ergosterol, or is only produced by the subsequent action of heat.

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The Integration of Plant Behaviour. III.—The Effect of Gravity on the Growth of Roots.

By Sir FREDERICK KERBLE, C.B.E., ScD., F.R.S., M. G. Nelson, M.A., and R. SNOW, M.A.

(Received April 7, 1931.)

The question whether the transverse stimulus of gravity affects the rate of growth of various parts of plants has been often investigated, but with results that are in part very contradictory. For a convenient discussion of the results obtained up to 1925, reference may be made to a paper by Gradmann (1925, p. 237). More recently Dolk (1929) has obtained automatic records of the growth of *Avena* coleoptiles when rotated on the horizontal axis of the clinostat and when placed vertical. His records, which seem quite free from objection, show so uniform a rate of growth that it must be concluded that in these plant organs in these conditions the change from the vertical to the horizontal position, or *vice versa*, makes no difference to the rate of growth. Cholodny also has obtained evidence that in hypocotyls of *Lupinus* and *Helianthus* (1929) and in coleoptiles of *Avena sativa* (1930), the rate of growth is not affected by their position in relation to gravity.

Dolk and Cholodny interpret these results in favour of the Cholodny-Went theory of geotropism, according to which the geotropic curvatures of roots and coleoptiles are brought about by growth-substances continually secreted by the tips, which become re-distributed between the upper and lower sides under the stimulus of gravity, though their total quantity is supposed not to be changed. Now the writers regard this theory favourably, but none the less it appears to them that even if this re-distribution does take place, and even if it suffices to account for the geotropic curvatures, it remains possible that in some plant organs at least the transverse stimulus of gravity may also alter the total quantity of growth-substance. This point has been emphasised by Gradmann in an interesting discussion (1930, p. 596). Actually it is well known that in the nodes of grasses the transverse stimulus of gravity does alter the rate of growth. For nodes that have completed their growth may start growing again if placed horizontal.

In the experiments to be reported here, the effect of gravity on the growth

of roots was investigated. The measurements were made after longer periods than those of Cholodny and Dolk, who did not continue their experiments for more than $2\frac{1}{2}$ hours. Our experiments were performed upon the main roots of seedlings of *Zea mays*, of a pure line called Red Cob, kindly supplied by Messrs. Sutton of Reading, and the plan was simply to compare the rate of growth of horizontal roots with that of vertical roots. The seedlings were germinated in sawdust and were kept both before and during the experiments in an electric thermostat at a temperature of 20° C. When the roots were ready for measurement the seedlings were transferred to saturated air, and were pinned in the required positions to strips of cork at the bottom of large glass vessels. The vessels contained also a shallow layer of water at the bottom and were closed with a lid.

In order to reduce as far as possible the errors due to differences in the rates of growth of different roots, the following method was adopted. Large numbers of seedlings were grown in batches of the same age, and the rates of growth of their roots were measured during a preliminary period or periods. Pairs of seedlings were then selected which had grown at almost exactly the same rates, and were also of about the same length, and one seedling of each pair was arranged with its root horizontal, and the other with its root vertical. A preliminary testing of this kind was found to make the results much more consistent.

In the first series of experiments, intact roots were used. Naturally those placed horizontal curved downwards. In 3 or 4 hours they curved through about 60° , but then, for the most part, curved no more. After this time, therefore, the transverse component of the stimulus of gravity acting upon the roots was only about half as strong as when the roots were horizontal. But for a large part of the first period of measurement, which was from $4\frac{1}{2}$ to 7 hours, the horizontally arranged roots were exposed to nearly the full transverse effect of gravity, for their curvatures only began to be considerable after 2 or 3 hours. For the purpose of measuring the curved roots, their outline was first traced on paper. A thread was then laid along the middle line of the drawing and transferred to a graduated scale.

The following table shows the results of the first experiment, the mean growth of the horizontally arranged roots in each series being expressed as a percentage of that of the vertical roots with which they were paired.

Experiment 1.—Zea mays. Growth of horizontally arranged intact roots, expressed as a percentage of that of the vertical roots with which they were paired.

Series.	Number of pairs.	Growth in first 4½ to 7 hours.	In next 19 to 22½ hours.
1	27	132.0	113.8
2	29	121.2	102.9
3	7	229.1	101.0
4	8	207.1	90.3
5	6	178.7	110.0
6	8	129.2	157.0
7	8	131.9	104.4
8	6	127.0	114.2
Total.....	99	Mean 144.0	110.3

From this table it can be seen that in the first period the horizontally placed roots grew considerably faster than the vertical roots in every series, and that in the second period, although they had curved through about 60° they still grew faster in every series but one.

Another experiment was performed in a similar manner on roots decapitated at 1 mm. behind the tip of the root cap. Of the horizontal decapitated roots, only about 7 per cent. curved downwards perceptibly. The following table shows the results expressed as before :—

Experiment 2.—Zea mays. Growth of horizontally arranged decapitated roots expressed as a percentage of that of vertical decapitated roots paired with them.

Series.	Number in pairs.	Growth in first 6 hours.	In next 18 hours.
1	5	185.5	129.4
2	13	108.6	146.1
3	9	132.0	115.0
4	5	112.0	109.0
5	5	95.0	113.0
Total.....	37	Mean 123.3	126.7

This table shows that the horizontally arranged roots again grew on the average considerably faster than the vertical roots in both periods of measurement. The difference was less than in experiment 1 in the first period, but greater in the second period, the reason being probably that the decapitated roots did

not curve, but remained horizontal. It is a point of interest that decapitated roots appear to be accelerated in growth by the transverse stimulus of gravity, although this stimulus seldom causes them to curve.

In two further experiments comparisons of the rates of growth in the horizontal and vertical positions were made with maize roots, which, for various reasons, were treated in more complicated ways. In one of these experiments the roots were decapitated as before, and then had tips of maize coleoptiles placed upon them, and in the other experiment they were similarly decapitated, but had their own tips replaced upon them. The horizontally arranged roots mostly curved downwards, though less rapidly and less far than intact roots. The two following tables give the results expressed as before :—

Experiment 3.—*Zea mays*. Growth of horizontally arranged decapitated roots, with coleoptile tips placed upon them, as a percentage of that of similarly treated vertical roots.

Series.	Number of pairs.	Growth.	Period of measurement.
1	6	304	hours. 18
2	20	121	24
3	15	126	22
Total.....	41	Mean 149.6	—

Experiment 4.—*Zea mays*. Growth of horizontally arranged decapitated roots with their own tips replaced, as a percentage of that of similarly treated vertical roots.

Series.	Number of pairs.	Growth in first 5 to 6 hours.	In next 15 to 16 hours.
1	16	164	169
2	11	109	93.9
Total.....	27	Mean 141.8	138.3

From Tables 3 and 4 it can be seen that the horizontally arranged roots again grew the faster except in the second series of experiment 4, in the second period of measurement, in which they grew more slowly.

The rates of growth in width also of decapitated maize roots were compared in the vertical and horizontal positions. Pairs of roots were used which were

found to be growing in length at equal rates, while still vertical, as in the previous experiments. They were then decapitated at 1 mm. from the tip of the root cap, and their diameter was measured close to the cut ends with a horizontal microscope with micrometer eye-piece. The following table shows the growth in width of the horizontal roots expressed as a percentage of that of the vertical roots :—

Experiment 5.—Zea mays. Growth in width of decapitated horizontal roots as a percentage of that of the decapitated vertical roots paired with them.

Series.	Number of pairs.	Growth in 6 to 8½ hours.
1	6	200
2	6	241
3	6	180
Total	18	Mean 197

This table shows that growth in width also was accelerated in the horizontal position, and more strongly so than growth in length.

Experiments on growth in length similar to experiments 1 and 2 were performed on roots of pea seedlings also. The two following tables show the results obtained with intact and decapitated roots of peas :—

Experiment 6.—Pisum sativum. Growth of horizontally arranged intact roots, expressed as a percentage of that of the paired vertical roots.

Series.	Number of pairs.	Growth in first 6 to 7 hours.	In next 16 to 18 hours.
1	9	71	110
2	5	108	92
3	7	116.3	103.2
4	4	184.8	118.4
5	6	124.4	68.4
6	8	148.3	104.1
7	7	126.6	120.3
Total.....	46	Mean 118.0	102.7

Experiment 7.—*Pisum sativum*. Growth of horizontally arranged decapitated roots expressed as a percentage of the paired vertical roots.

Series.	Number of pairs.	Growth in first 5 to 6 hours.	In next 16 to 18 hours.
1	7	98	106
2	8	78·2	82·9
3	6	135·4	67·3
4	8	108·6	77·9
5	7	146·8	130·1
Total.....	36	Mean 110·7	92·8

The above two tables show that in pea seedlings also the horizontally placed roots for the most part grew faster than the vertical roots. The results with pea roots, though less regular than those obtained with maize, nevertheless lend definite support to the conclusions to be drawn from the experiments with maize roots.

The results obtained with maize seem quite sufficient to show that the rate of growth of the root is accelerated in the horizontal position. This conclusion was quite unexpected, and even though correct, the writers, for the reasons already given, do not consider that it is in conflict with the Cholodny-Went theory of geotropism.

Summary.

In maize seedlings, the main roots, whether intact or decapitated, were found, when placed horizontal, to grow more rapidly than similar vertical roots, both in length and in width.

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The Development of Various Strains of Leishmania in Chinese Sandflies.

(A Report of the Kala Azar Commission of the Royal Society.)

By EDWARD HINDLE,* Wellcome Bureau of Scientific Research.

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A study of the development of the Chinese strain of *Leishmania donovani* in *Phlebotomus chinensis* (= *P. major* var. *chinensis*) and *P. sergenti* var. *mongolensis* (subsequently referred to as *P. mongolensis*) has shown that although in both species the *Leishmania* develops into the flagellate stage, it is only in *P. chinensis* that the flagellates become attached to the lining of the anterior part of the crop, and only in this species that they ever invade the pharynx and buccal cavity. In *P. mongolensis* the flagellate forms remain confined to the hinder part of the stomach, do not become attached to the lining and, moreover, persist only as long as undigested food is present (Hindle, 1928).

It seemed of interest, therefore, to study the development of other strains of *Leishmania* in these two species of sandflies, to see whether any specific correlation existed between them.

Through the kindness of Dr. Marshall Hertig three non-Chinese strains of *Leishmania* were obtained from the Peking Union Medical College. These strains were as follows :—

1. An Indian strain of *L. donovani*, originally isolated from kala azar cases at Golghat, Assam, by Major Shortt and his colleagues. This strain had been maintained in cultures for at least one year before being received.
2. A Tunis strain of *L. infantum*, originally obtained in culture from Dr. Nicolle, and which had been maintained in culture for several years.

The peculiar feature of the infection produced in hamsters by this strain was the occurrence of a visceral infection which died out, and the

* The observations recorded in this paper were begun in China and continued, by kind permission of the Beit Trustees, whilst the author was holding the Beit Memorial Fellowship in Tropical Medicine.

subsequent localisation of the parasite in the testes and the neighbourhood of the joints, where it produced marked swelling. (Hindle and Thomson, 1928).

3. A strain of *L. tropica* isolated by Dr. C. W. Young on April 1, 1926, from cases of oriental sore in Baghdad, and subsequently maintained in culture.

Chinese hamsters, both *Cricetulus griseus* and *C. triton*, were inoculated with each of these strains, and sandflies fed on infected animals and dissected after various intervals. The methods employed for the feeding experiments were identical with those detailed in our previous reports (Patton and Hindle, 1927). In addition, sandflies were fed artificially on suspensions of *Leishmania*, by using the feeding apparatus recommended by the Hertigs (1927).

Experiments with an Indian Strain of L. donovani.

Animal Experiments.

The cultures received from Dr. Hertig were at the seventh passage in sub-culture since their isolation from kala azar cases in Assam, approximately one year previously.

Twelve hamsters (Nos. 767 to 778) were each inoculated intraperitoneally with 0.2 c.c. of a saline suspension from a 12-day old culture containing numerous flagellates. Five days later all the hamsters were re-inoculated with a similar dose from a 17-day culture. Four of these hamsters died at intervals of 2 to 10 weeks after being inoculated and were negative.

No. 773 died after 13 weeks, and although the spleen was not enlarged—an almost constant feature of infections with the Chinese strain—films of the liver and spleen showed the presence of large numbers of *Leishmania*.

No. 774 was killed after 14 weeks and showed a moderate infection of the spleen and liver.

No. 770 died after 4 months with a slightly enlarged spleen and films of the liver and spleen were positive.

No. 769 was killed after 4½ months and showed no signs of infection and organ films were negative.

No. 772, also killed after 4½ months, had an enlarged spleen and films of the liver, spleen and testis showed an extremely scanty infection; the bone marrow was negative.

No. 776 was killed after 6½ months and had a greatly enlarged spleen.

The liver and spleen contained a moderate infection, but the bone marrow was negative.

No. 775 was killed after $7\frac{1}{2}$ months and had a greatly enlarged spleen but had only a moderate infection of the liver, and scanty infections of the spleen and kidney. The bone marrow was negative.

No. 777 was also killed after $7\frac{1}{2}$ months and showed a greatly enlarged spleen. Films of the liver, spleen and bone marrow showed very rare parasites.

In this original experiment, therefore, 7 out of 12 hamsters became infected, but only one of them (No. 773) showed a degree of infection in any way comparable with the type of infection resulting from inoculation with flagellates of the Chinese strain of kala azar (Hindle and Patton, 1926).

Two giant hamsters, *Cricetulus triton*, were also inoculated from the same suspension of flagellates used in the above experiments.

No. 779 was killed 7 months later and showed no signs of infection.

No. 780, killed 3 weeks later, had a greatly enlarged spleen; films showed a moderate infection in the spleen, and very scanty infections of the liver and bone marrow.

Subinoculations were made from these infected animals into 16 *Cricetulus griseus* (Nos. 885 to 894 and 903 to 908); and 4 *C. triton* (Nos. 901, 902, 909 and 910), and with one exception all these hamsters showed typical infections with *Leishmania* when examined 3 to 4 months after inoculation. The results obtained, therefore, by the inoculation of the non-flagellate stage of this Indian strain, direct from one hamster to another, resemble those obtained with the Chinese strain.

Results of Feeding Sandflies on Hamsters Infected with an Indian Strain of L. donovani.

The above-mentioned hamsters were used for transmission experiments with two species of sandflies, viz., *Phlebotomus chinensis* and *P. mongolensis*. In all cases the methods employed were those described in previous reports of our Commission (Patton and Hindle, 1927; Hindle, 1928). The results of these experiments are summarised in the following tables:—

Table A.—Experiments with *Phlebotomus chinensis*.

Number of experiment and date when fed.	Number of flies fed.	Temperature.	Number of hamster on which flies fed.	Number of days between feeding and dissection.	Number of flies dissected.	Results of dissection and remarks.
160 (18.6.37)	3	25	779	5	3	All negative.
168 (20.6.37)	6	25	775	4 6 7	2 1 3	Both negative. Negative. One negative; two heavily infected in mid-gut, but only in hinder part of mid-gut. One of these flies was refed the day previously on hamster S.77, and the contents of both were inoculated into hamster S.79. Both these hamsters were negative when examined 6½ months later.
193 (24.6.37)	17	25	775	3 5 6 7	3 2 11 1	All negative. One negative; other heavily infected and contents inoculated into hamster S.87. Six negative; four moderately, and one heavily infected, but not in anterior part of mid-gut. Contents inoculated into hamster S.96. Both this hamster and S. 87 were negative when examined after 2 and 6 months respectively. Negative.
269 (1.7.37)	4	25	775	2 4	2 2	Both negative. One negative; one slightly infected. Contents inoculated into hamster S.169, which was negative when examined 6½ months later.
275 (16.7.37)	28	26	775	4 5	14 3	Eleven negative; three very slightly infected. All negative.

Table B.—Experiments with *Phlebotomus sergenti* var. *mongolensis*.

Number of experiment and date when fed.	Number of flies fed.	Temperature.	Number of hamster on which flies fed.	Number of days between feeding and dissection.	Number of flies dissected.	Results of dissection and remarks.
143 (15.6.27)	18	° C. 25	775	3	1	Positive in mid-gut.
144 (15.6.27)	19	25	776	4	12	Five negative; seven positive in mid-gut. Contents inoculated into hamster S.41 which was negative 6 months later.
145 (15.6.27)	7	25	777	1	1 } 13 }	All negative.
159 (18.6.27)	34	25	888	3	7	All negative.
160 (18.6.27)	5	25	779*	4	31	Twenty-nine negative; two very slightly infected.
161 (18.6.27)	15	25	780*	4	4	All negative.
162 (18.6.27)	22	25	889	4	8	Seven negative; one slightly infected.
223 (28.6.27)	34	25	885	4	2	Both negative.
225 (29.6.27)	34	25	886	4	27	Twenty-four negative; three positive. Contents inoculated into hamster S.109, which was negative when examined after nearly 7 months.
226 (29.6.27)	39	25	883	4	31	All negative.
227 (29.6.27)	29	25	889	4	31	Twenty-nine negative; two well infected. Contents inoculated into hamster No. S.113, which was negative when examined nearly 7 months later.
228 (29.6.27)	30	25	890	4	26	Twenty-one negative; five slightly infected. Contents inoculated into hamster No. S.116, which was negative when examined nearly 7 months later.
		25		4	16	All negative.

Discussion of Results.

The results of these experiments clearly indicate that the Indian strain of *Leishmania* does not develop in *P. chinensis* and *P. mongolensis* with the same readiness as Chinese strains of this parasite.

In the case of *P. chinensis* a total of 47 flies were dissected of which 35 were negative and 12 positive. With three exceptions, however, these positive flies showed very scanty infections, quite unlike the type of infection resulting when these sandflies are fed on a Chinese strain.

In every instance the flagellates were free in the hinder part of the stomach, and there was no suggestion of the anterior development and attachment of the flagellates to the wall of the gut, which is such a marked feature in the case of the Chinese strain. The infection rate with the Indian strain is approximately 25 per cent., as compared with approximately 78 per cent. in a corresponding series of experiments with a Chinese strain (Patton and Hindle, 1927).

Moreover, a very heavily infected hamster (No. 775) showing parasites in the subcutaneous tissue, was used for four out of the five feeding experiments recorded in Table A, therefore the conditions were favourable for the production of a high infection rate, had the sandfly been a suitable host. The results of the inoculation of these flagellates into four hamsters and the feeding experiment of S.77 gave uniformly negative results, but even with Chinese strains there is only a comparatively small percentage of infections, therefore these results are too few to be of any significance.

The results of the feeding experiments with *P. mongolensis* on 21 different hamsters infected with the Indian strain of kala azar, also indicate that this strain does not develop in this Chinese sandfly with the same readiness as the local strains of the disease. It will be seen that out of 464 sandflies dissected, mostly after an interval of only 4 days, flagellates were found in only 29 individuals, giving an infection rate of approximately 6 per cent., as compared with a rate of about 36 per cent. obtained with a Chinese strain of *Leishmania* (Patton and Hindle, 1927). The type of development was similar with both strains, the flagellates being confined to the hinder part of the stomach and never becoming attached to the wall of the alimentary canal. Six hamsters inoculated with the contents of these positive sandflies remained uninfected.

The conditions of the feeding experiments with these Chinese and Indian strains of kala azar were made to resemble each other as much as possible, and therefore, the marked difference in the infection rates in both species of

sandflies strongly suggests a biological distinction between the two strains. The Chinese strains of *Leishmania* readily develops in the two common local sandflies, *P. chinensis* and *P. mongolensis*, but the Indian strain, although showing some capacity for development in these sandflies, evidently does not find favourable conditions in them, as shown by the very low infection rate, and especially by the absence of any tendency to become attached to the lining of the gut, or of any development in the anterior part of the stomach of *P. chinensis*, which is such a marked feature with Chinese strains of the disease.

Experiments with a Tunis Strain of Leishmania infantum.

The results of attempts to infect hamsters with *L. infantum* have been recorded in a previous publication (Hindle and Thomson, 1928), and it is only necessary to state that the hamster is much less susceptible to this strain than to the Chinese and Indian strains of *L. donovani*. Some individuals, however, showed many parasites in the peripheral blood, and presumably in such cases any sandflies fed on them must have had an opportunity of becoming infected.

In every case the hamsters used in the following experiments were killed and examined for the presence of parasites, and any flies that had been fed on negative animals were eliminated. In addition to feeding on hamsters, one or two sandflies were fed artificially on cultures of this parasite, which, of course, were in the flagellate stage. The method used was that devised by the Hertigs (1927), in which the sandfly is held in position in a glass tube by a spilt cork, and the medium fed to the insect by means of a fine glass pipette placed over its proboscis. After a little practice flies were readily fed by this method, and in such cases one could be quite certain that any insect thus fed had ingested parasites.

The results of all feeding experiments with this strain, whether on hamsters or on artificial media, are summarised in the following tables :—

Table C.—Experiments with *Phlebotomus chinensis*.

Number of experiment and date when fed.	Number of flies fed.	Temperature.	Number of hamster on which flies fed.	Number of days between feeding and dissection.	Number of flies dissected.	Results of dissection and remarks.
M. 42 (7.8.26)	2	° C. 30	475	5	1	Negative.
M. 72 (21.8.26)	3	20	475	2 6	1 1	Negative. Negative.
148 (16.6.27)	10	25	805	2 4	2 8	All negative. All negative.
149 (16.6.27)	9	25	814	4	7	All negative.
232 (30.6.27)	11	25	581	3 4 5 6 7 8	3 3 2 1 1 1	Two negative; one heavily infected in mid-gut. Contents inoculated into hamster S.119, which remained uninfected. All negative. Both negative; one refed 2 days previously. Negative; refed 3 days previously. Negative. Negative.
277 (15.7.27)	10	25	581	4 5 6	4 2 4	All negative. Both negative. One negative; three positive with a very slight infection in the hinder part of the mid-gut. Contents inoculated into giant hamster S.176, which remained uninfected.
(29.3.27)	1	25	Rich culture	1	1	Negative.

Table D.—Experiments with *Phlebotomus sergenti* var. *mongolensis*.

Number of experiment and date when fed.	Number of flies fed.	Temperature.	Number of hamster on which flies fed.	Number of days between feeding and dissection.	Number of flies dissected.	Results of dissection and remarks.
S 37 (2.8.26)	40	30 °C.	475	1	3	All negative.
				2	6	Five negative; one positive (rosette forms). Surviving flies reled on hamster 476.
				3	6	Five negative; one positive. Contents inoculated into hamster 502, which remained uninfected.
				4	3	All negative. Survivors reled on hamster 475.
				5	11	Ten negative; one positive, showing a few flagellates. Contents inoculated into hamster 511, which remained uninfected.
				7	6	Five negative; one contained non-motile and degenerating flagellates.
S 48 (12.8.26)	40	20	475	2	4	All negative.
				3	12	All negative.
				4	10	Nine negative; one positive, three flagellates in mid-gut.
S 61 (17.8.26)	40	20	476	5	10	All negative.
				2	8	All negative.
				3	9	All negative.
S 70 (20.8.26)	27	20	475	4	10	Eight negative; two positive in mid-gut.
				3	8	All negative.
				4	4	Survivors reled on a normal hamster.
S 76	32	20	476	5	9	Seven negative; two positive with very scanty infection. Contents inoculated into hamster 591, which remained uninfected.
				2	9	All negative, but one fly contained a degenerating parasite.
				3	11	All negative. Survivors reled on a normal hamster.
				4	5	All negative.
				5	1	Negative.
				6	1	Negative.
				7	4	All negative.

Tab continued

Number of experiment and date when fed.	Number of flies fed.	Temperature.	Number of hamster on which flies fed.	Number of days between feeding and dissection.	Number of flies dissected.	Results of dissection and remarks.
S. 77 (30.8.26)	65	°C. 20	476	2 3 4	9 20 14	Seven negative; two positive. Contents inoculated into hamster 614, which remained uninfected. All negative. Thirteen negative; one positive. Contents inoculated into hamster 615, which remained uninfected. Survivors re-fed on a normal hamster. All negative. All negative. All negative.
S. 80 (6.9.26)	30	20	476	3 4	10 5	All negative. All negative.
148 (16.6.27)	19	25	805	4	12	All negative.
149 (16.6.27)	30	25	814	4	30	All negative.
171 (20.6.27)	53	25	581	4	38	Thirty-seven negative; one positive, very few flagellates in mid-gut.
172 (30.6.27)	74	25	582	4	58	All negative.
281 (16.7.27)	15	25	806	4	15	All negative.
286 (16.7.27)	7	25	807	4	7	All negative.

287 (18.7.27)	5	25	808	4	4	All negative.
292 (18.7.27)	17	25	810	3	12	All negative.
298 (19.7.27)	10	25	815	3	9	All negative.
301 (20.7.27)	18	27	817	3	14	All negative.
311 (22.7.27)	44	27	635	3	39	All negative.
320 (24.7.27)	35	28	747	3	25	All negative.
345 (6.8.27)	29	28	895	3	26	Twenty negative; six positive. Contents inoculated into hamster S.224 and S.225, which remained uninfected.
346 (6.8.27)	35	28	896	3	30	All negative.
348 (8.8.27)	39	28	899	3	33	All negative.
(21.3.28)	1	25	Culture	7	1	Negative. Twice refed on normal hamster.
(29.3.28)	1	25	Culture	1	1	Negative.

The results of these experiments indicate that both *P. chinensis* and *P. mongolensis* are not favourable hosts for the development of this strain of *Leishmania infantum*. In the case of *P. chinensis*, 41 flies fed well on four different hamsters, each well infected with *L. infantum*, yet only four individuals showed the presence of flagellates in the stomach, and three of these had only very scanty infections. Also one fly that was fed artificially with a rich culture of this strain showed no signs of flagellates when dissected the following day.

In the case of *P. mongolensis*, 573 flies were fed on 17 different infected hamsters, and of these only 18 showed the presence of living flagellates when examined after suitable intervals, although another two flies contained degenerating flagellates.

In addition, two flies were fed artificially on a culture of the flagellates and dissected respectively 1 and 7 days later. Both were negative.

The contents of most of the infected sandflies were inoculated into normal hamsters, none of which showed any signs of infection.

Experiments with a Bagdad Strain of Leishmania tropica.

This strain was originally isolated on April 1, 1926, by the late Dr. C. W. Young from a case of Oriental Sore in Bagdad, and when received had been subcultured six times. Attempts were made to infect hamsters by the inoculations of these cultures, but the results show that very few successes were obtained.

Five young male hamsters (Nos. 781 to 785) each received an intratesticular inoculation of a rich culture and 3 days later were all reinoculated intraperitoneally with another culture of the same strain. These animals were killed and examined after intervals varying from 1 to 7 months, and only one (No. 781), killed after nearly 5 months, showed any signs of infection. In this animal a few parasites were found in the testes, but the liver, spleen, bone-marrow and abdominal lymphatics were negative.

Five female hamsters (Nos. 786 to 790) and two males (Nos. 791 and 792) were inoculated intraperitoneally with the same suspension used above, and the animals killed and examined at intervals varying from 5 to 7 months, except one which died after 3 weeks. None of these animals showed any signs of infection.

Two hamsters (Nos. 793 and 794) were exposed to infection by shaving the nose and rubbing a culture into the excoriated surface. They were examined at intervals up to 7 months later without showing any signs of infection. Two

giant hamsters, *C. triton*, and a 4 weeks old puppy were also inoculated with negative results.

It will be seen, therefore, that out of 14 small hamsters, two large ones, and a puppy, inoculated in various ways with these cultures, only one individual (No. 781) became infected. In this animal the infection was confined to the testes.

Three male hamsters (Nos. 915 to 917) were each inoculated intratesticularly with a saline suspension of the testes of hamster No. 781. Eleven weeks later these animals were killed and examined. No. 915 showed a slight infection of the right testis, whilst the left testis and all other organs were negative; No. 917 showed a very heavy infection in the right testis, which was atrophied, a slight infection in the left testis, and all other organs were negative; No. 916 showed no signs of any parasites in its organs, but the left testis was atrophied and caseous.

Three female hamsters (Nos. 918 to 920) inoculated intraperitoneally with the same suspension showed no signs of infection when examined at the same time as Nos. 915 to 917.

The results of these few experiments suggest that the testis is the only organ of the hamster in which this strain of *L. tropica* is able to develop, and that the intratesticular method of inoculation is the one most likely to succeed (see also Wenyon and Hindle, 1928).

Experiments with Sandflies.

In view of the localisation of the parasites to the testis it was not to be expected that sandflies fed on these hamsters would show any development of flagellates. One batch of *P. mongolensis*, however, was fed on hamster No. 915 and 22 flies dissected after an interval of 3 days. They were all negative.

The results of artificial feedings, although few in number, indicate that *P. mongolensis* may possibly be a suitable host for the development of *L. tropica*, but further observations are necessary. The results of our experiments are as follows :—

March 29, 1927.—One *P. mongolensis* and one *P. chinensis* were each well fed on a culture of *L. tropica* and kept at 25° C. The following day they were dissected and the first species was negative, whilst the other contained only one or two flagellates which were obviously degenerating.

April 12, 1927.—Two *P. mongolensis* and one *P. chinensis* were each fed on a rich culture of *L. tropica* and kept at 24° C. The following day one of each species was dissected and both were negative. On the following day the

surviving *P. mongolensis* was killed and dissected. Numerous flagellates were present in the stomach, but none in the anterior cardia.

April 15, 1927.—Two *P. mongolensis* were fed on a culture of *L. tropica*. Three days later one was examined and found to be negative, whilst the other, dissected on the fourth day, contained a few flagellates in the stomach.

It will be seen, therefore, that out of five *P. mongolensis* fed on cultures of *L. tropica*, two became infected; whilst of two *P. chinensis*, one showed flagellates the following day and the other was negative.

Experiments with Phlebotomus squamirostris (= taianensis) and a Chinese Strain of Leishmania donovani.

In nature, this species of sandfly feeds on cold-blooded animals, and although we succeeded in feeding some individuals on hamsters, we were never able to get any to feed on human subjects. Consequently this species can have no significance in the transmission of kala azar, as it would never be exposed to infection. It seemed of interest, however, to see whether human *Leishmania* would develop in such a species of sandfly.

The earlier experiments were made by means of artificial feeding and are as follows :—

June 2, 1927.—One *P. squamirostris* was fed on a culture of a Chinese strain of *L. donovani* and kept at 25° C. Four days later it was dissected and found to be negative.

June 6, 1927.—Three *P. squamirostris* were fed on a suspension of the spleen and liver of hamster No. 173, heavily infected with a Chinese strain of kala azar. Two days later they were dissected. One was negative, but the other two were heavily infected with flagellates in the stomach.

The results of this experiment clearly show that the Chinese strain of *Leishmania* is capable of developing to the flagellate stage in *P. squamirostris*, but curiously enough all attempts to infect them by feeding on infected hamsters gave uniformly negative results. A total of 22 of these sandflies were fed on five different hamsters, all proved infective to *P. chinensis* and *P. mongolensis*. These flies were dissected at intervals ranging from 1 to 7 days, yet no trace of *Leishmania* could be found in any of them. It is difficult to explain the negative results of these experiments, as in most instances either *P. chinensis* or *P. mongolensis* were fed on the infected hamster at the same time as the *P. squamirostris*, and yet whilst some of the former invariably showed the development of flagellates, the latter were uniformly negative.

The extremely rapid digestion in this species, resulting in the gut becoming empty within 24 hours, may explain some of the negative results, but this is not a full explanation, in view of the positive results obtained when the flies were fed artificially.

Discussion of Results.

The results obtained by feeding two species of Chinese sandflies, *P. chinensis* and *P. mongolensis*, on hamsters infected with three different strains of *Leishmania*, are summarised in the following tables :—

Results of Feeding *P. chinensis* on Hamsters Infected with Different Strains of *Leishmania*.

Strain of <i>Leishmania</i> .	Number of flies fed.	Number showing flagellates.	Percentage.
Chinese	157	122	78
Indian	47	12	25.5
<i>L. infantum</i>	41	4	9.8

Results of Feeding *P. mongolensis* on Hamsters Infected with Different Strains of *Leishmania*.

Strain of <i>Leishmania</i> .	Number of flies fed.	Number showing flagellates.	Percentage.
Chinese	1170	430	36
Indian	464	28	6
<i>L. infantum</i>	573	18	3.14

When making any comparison between these results, it is important to remember the possibility of individual variation in the infectivity of hamsters inoculated with the same strain, as well as the different types of infections obtained in these animals when infected with different strains.

In order to eliminate, as far as possible, differences due to individual variations in the susceptibility of the vertebrate host, sandflies were fed on several infected hamsters, so that other conditions being equal, any difference in the infection rates would be of significance. Differences in the general type of infection produced by different strains, however, can only be met by feeding sandflies artificially with cultures or suspensions of the various parasites. Thus the fact that sandflies fed on a hamster infected with *L. tropica* never

showed any flagellates in the alimentary tract is almost certainly due to the peculiar localisation of the parasites to the testes of infected animals. As a result a sandfly feeding on such an animal would not ingest any parasites and consequently would not become infected, even though the gut of the fly might furnish ideal conditions for their development.

The other three strains all agree in producing a general type of infection in the hamster, but whereas in the case of the Chinese and Indian strains of *L. donovani*, the infections are progressive and usually remain generalised, *L. infantum*, on the other hand, after first producing a generalised infection of the viscera, afterwards tends to be restricted to certain regions of the body, especially the neighbourhood of certain joints, and in the testes. Sandflies fed on hamsters infected with any of these three strains would have a reasonable chance of ingesting parasites, since they all invade the blood circulation.

Assuming that on an average the sandflies had similar chances of becoming infected, it is obvious that there are very marked differences in the capacity for development shown by these three strains.

In the first place, the only sandflies that ever showed flagellates in the cardia and pharynx were *P. chinensis* that had fed on hamsters infected with Chinese strains of *Leishmania donovani*. *P. mongolensis* fed on hamsters infected with the same strain, and with few exceptions on the same individuals, never showed any invasion of the anterior region of the alimentary canal, the flagellates being confined to the stomach. Similarly, both these species of sandflies after feeding on hamsters infected either with an Indian strain of kala azar, or with *L. infantum*, merely showed flagellates free in the stomach.

The results suggest that although there is a general capacity on the part of *Leishmania*, after being ingested by various species of *Phlebotomus*, to develop into the flagellate stage, it is only when there is some biological relationship between the two that development proceeds further. When this occurs the flagellates become attached to the lining of the gut, especially in the cardia, and invade the pharynx, from where they may eventually extend into the proboscis. The writer's views, therefore, are in full agreement with those advanced by Adler and Theodor (1927), based on the results of their feeding experiments with *P. papatasi* on a strain of *L. brasiliensis*, two strains of *L. infantum* and strains of *L. tropica*.

When making feeding experiments with various species of sandflies to determine which is the most probable carrier of any particular strain of *Leishmania*, attention should be concentrated on any which show this particular type of development, for the mere production of flagellates in the gut of an insect,

especially if unaccompanied by any obvious multiplication in their numbers, is of little significance. Where a definite multiplication of the flagellates occurs, as in the case of the Chinese strain of *L. donovani* in the gut of *P. mongolensis*, it is possible that there is an approximation to the biological conditions necessary for complete development and invasion of the proboscis. In this connection it is of interest that the partial development in *P. mongolensis* was much more easily obtained with the local Chinese strain of *Leishmania* than with an Indian strain, the infection rates being respectively 36 per cent. and 6 per cent.

With *L. infantum* the infection rate is even lower, 3.14 per cent., but this may be due to the lesser susceptibility of the hamster.

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Nitrite Formation by Soil Bacteria, other than Nitrosomonas.

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Introduction.

The biological nature of nitrification in soil was first established by Schlössing and Müntz (1877). Warington (1878, etc.) at Rothamsted and numerous other investigators elsewhere advanced considerably the knowledge of this process. Winogradsky (1890) first succeeded in isolating two species of bacteria, one capable of oxidising ammonia into nitrite and the other nitrite into nitrate; he further showed that nitrification in soil can take place only through the activity of a very limited group of autotrophic bacteria possessing the peculiar property of growing exclusively in an inorganic medium having a very pronounced alkaline reaction.

In 'Nature' Cutler (1930) reported that several different bacterial strains capable of oxidising various ammonium salts to nitrite had been isolated from different sources. A description of some of these bacteria occurring in soil, together with their physiological reactions, is given in this paper.

As a result of investigations on the growth of bacteria on silica gel plates, some species were found which were capable of oxidising ammonium salts to nitrite; but which did not resemble the *Nitrosomonas* group of organisms in that they grew freely on nutrient agar medium containing lemco and peptone. This was interesting, especially in view of the observation made by Winogradsky (1890, 1891) that the most striking characteristic of his nitrite producers, *Nitrosomonas* or *Nitrosococcus*, was their marked repugnance to organic substances.

There are, however, scattered references in the literature to organisms capable of oxidising ammonia to nitrite and nitrate, and of growing in media containing organic as well as inorganic nutrient substances.

Stützer and Hartleb in 1897 isolated *Nitrosomonas* by using magnesium-ammonium-phosphate agar plates. Silicic acid gel was also used by Stützer (1901), but did not give satisfactory results.

Beddies in 1899 reported the isolation of nitrifying organisms; these were

not very sensitive to high concentrations of organic matter and the presence of humus in the medium aided the growth.

Fremelin in 1903 isolated a nitrite-forming organism, and showed that this bacterium would grow on bouillon. In 1914, using agar, he found that the presence of broth or urine increased the activity of this micro-organism; and recently (1930) he has again shown that it is capable of growing in the presence of organic substances.

Makrinoff (1909) isolated a nitrite-forming organism by employing gypsum plates and found that the presence of soil did not prove toxic; a result also obtained by Gibbs (1909) when cultivating *Nitrosomonas*.

Mishustin (1926) found two spore-forming bacteria in soil which produced nitrites in media containing organic nitrogenous compounds, but not in inorganic media containing ammonium salts.

Runov (1926) obtained two species from enrichment cultures, one of these produced nitrites from organic nitrogen compounds and from ammonia in the presence of organic substances. Neither organism grew on Winogradsky's medium and the author concluded that there were many bacteria in nature capable of forming nitrites in media containing various organic substances.

The diagnostic characters given in these cases were, however, insufficient for identification purposes.

Experimental.

In the course of work on the isolation of *Nitrosomonas*, numerous bacterial colonies were picked from silica gel plates for further investigation. These were inoculated into Winogradsky's liquid medium containing excess of ammonium sulphate but no magnesium carbonate.* Periodic nitrite determination by the Griess-Ilosva method showed that these organisms were capable of oxidising ammonium sulphate into nitrite, and the quantities of nitrite formed compared very favourably with those found in a similar culture inoculated with fresh soil.

Portions of these enrichment cultures were plated on Thornton's agar and white colonies appeared after 4 days. A number of these colonies were picked off and inoculated on to Thornton's agar slopes, where a copious growth soon took place. Six strains, A, B, C, D, E and F were selected for further work.

In order to test whether the presence of sugar exerted any effect on the

* It was found from preliminary experiments that magnesium carbonate was not essential to the growth or activity of these organisms.

production of nitrite by these organisms the following mineral salt medium was made :—

	Per cent.
$(\text{NH}_4)_2\text{SO}_4$	0.1
NaCl	0.06
CaCl_2	0.002
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.0005
KH_2PO_4	0.03

The p_{H} value after autoclaving was brought to 7.3 by the addition of NaOH and 0.1 per cent. sucrose was added to some of the medium.

50 c.c. portions of these two media were distributed into an equal number of 250 c.c. Erlenmeyer flasks which were inoculated with the bacteria.

The amounts of nitrite produced in each of the cultures are shown in Table I.

Table I.—Nitrite Nitrogen in milligrammes per litre produced by the different species in mineral salts medium—both in the presence and absence of sugar.

Days.	Sugar.	A.	B.	C.	D.	E.	F.	Control.
2	+	0.13	Trace	Nil	Nil	Trace	0.13	Nil
	—	0.13	..	..	Trace	..	Nil	..
5	+	0.25	0.15	0.15	Nil	0.13	Trace	..
	—	0.13	Trace	Nil	..	Nil	Nil	..
8	+	0.5	0.25	..	0.13	0.25	0.25	..
	—	0.13	0.13	Trace	Nil	0.13	Trace	..
12	+	0.75	0.75	0.25	0.25	0.25	0.5	..
	—	0.25	0.5	Trace	Nil	Nil	Nil	..
17	+	1.25	1.0	0.50	0.25	0.5	0.5	..
	—	0.25	0.5	0.25	0.25	Nil	Trace	..
23	+	1.0	1.0	0.75	0.50	1.0	1.0	..
	—	0.50	0.75	0.25	0.25	0.25	Nil	..
28	+	0.5	0.5	0.25	0.75	0.75	1.0	—
	—	0.5	0.5	Nil	0.25	0.50	0.5	—

It is evident that in the presence of sugar these organisms produced more nitrite than in its absence, which is of interest considering that no growth occurs in sugar solutions (see p. below). In all the following investigations, therefore, the mineral salt medium containing 0.1 per cent. sucrose was used unless otherwise stated.

To ensure that only pure cultures were being investigated repeated platings and pickings off of single colonies were made; and the pure strains finally obtained were known as A₁, A₂, B₂, C₂₁, C₂₂, D₂₃, E and F₁₁.

The nitrite produced by each of these strains from ammonium phosphate, chloride, lactate, acetate and sulphate is shown in Table II. The nitrogen equivalent of each of the added salts amounted to 0.02 gm. per 100 c.c. medium.

Table II.—Nitrite formed by species A₁, A₂, B₂, C₂₁, C₂₂, D₂₃, E and F₁₁ from various ammonium salts. (Nitrite-nitrogen in milligrammes per litre.)

Ammonium salts.	Bacteria.	Days.						
		2.	4.	6.	9.	11.	13.	16.
Phosphate	A ₁	0.125	Nil	Nil	Trace	Trace	Trace	Trace
	A ₂	Nil	"	Trace	0.125	0.25	0.25	0.125
	B ₂	"	Trace	"	Nil	Nil	Nil	Nil
	C ₂₁	"	Nil	0.25	0.5	0.25	Trace	Trace
	C ₂₂	"	"	Nil	Nil	Nil	"	Nil
	D ₂₃	"	"	Trace	0.5	0.5	0.5	0.125
	E	"	"	0.125	0.25	0.25	Nil	Nil
	F ₁₁	"	0.125	0.25	0.5	0.5	"	0.125
Chloride	A ₁	"	Nil	Nil	0.25	0.5	Trace	Trace
	A ₂	"	0.125	0.25	0.5	0.25	0.75	0.5
	B ₂	"	Trace	Nil	Nil	Nil	Trace	0.125
	C ₂₁	"	"	"	"	"	"	Nil
	C ₂₂	Trace	Nil	"	"	Trace	0.25	0.25
	D ₂₃	Nil	0.125	0.125	0.25	0.25	0.25	0.25
	E	"	Nil	Nil	Nil	Nil	Nil	Trace
	F ₁₁	"	"	"	Trace	"	"	Nil
Lactate	A ₁	"	Trace	0.125	0.75	0.75	0.75	0.5
	A ₂	"	0.125	0.5	0.5	0.5	0.75	0.5
	B ₂	"	Trace	0.25	0.5	0.25	Nil	Nil
	C ₂₁	"	"	Trace	Nil	Nil	"	"
	C ₂₂	"	Nil	0.125	Trace	Trace	Trace	Trace
	D ₂₃	Trace	0.125	0.5	0.5	0.25	0.25	0.25
	E	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	F ₁₁	"	"	"	"	"	"	Trace
Acetate	A ₁	"	"	Trace	"	"	"	Nil
	A ₂	Trace	"	0.125	0.25	0.25	0.25	0.125
	B ₂	Nil	"	Nil	Nil	Nil	Nil	Nil
	C ₂₁	"	"	"	Trace	Trace	"	"
	C ₂₂	"	"	0.125	Nil	Nil	"	"
	D ₂₃	"	"	Nil	"	0.25	0.25	Trace
	E	"	"	"	"	Trace	Trace	Nil
	F ₁₁	"	0.125	0.125	"	Nil	Nil	"
Sulphate	A ₁	"	Trace	Nil	0.25	Trace	"	"
	A ₂	"	0.125	0.125	0.25	0.25	"	"
	B ₂	"	Nil	0.125	0.25	0.5	0.5	0.25
	C ₂₁	Trace	Trace	Nil	0.125	Trace	Trace	Nil
	C ₂₂	Nil	Nil	"	0.125	0.125	Nil	"
	D ₂₃	Trace	"	"	0.5	0.5	0.25	Trace
	E	"	0.125	0.25	0.75	0.5	Nil	"
	F ₁₁	Nil	Trace	0.25	0.5	0.25	"	"

It will be seen that ammonium phosphate, chloride, lactate and sulphate can be oxidised into nitrite by these bacteria, but with ammonium acetate the oxidation is not very marked. The yields of nitrite are greatest with lactate and sulphate of ammonia, the former giving somewhat better results than the latter excepting in the cases of E and F₁₁.

It is interesting to note that in the majority of the cultures the amounts of nitrite diminished gradually, and the following experiments were carried out in order to test if the organisms were capable of utilising nitrite.

Sulphate of ammonia was replaced by sodium nitrite in the mineral salt medium containing 0.1 per cent. sucrose. The amount of nitrite in the medium corresponded to 1.8 mgm. of nitrogen per litre, and 50 c.c. portions of the medium were distributed into 250 c.c. Erlenmeyer flasks which were subsequently inoculated.

The results of the nitrite estimations are given in Table III.

Table III.—Nitrite Utilisation by A₁, A₂, B₂, C₂₁, C₂₂, D₂₂, E and F₁₁. (NO₂-N) originally present : 1.8 mgm. per litre.)

Days.	NO ₂ -N in milligrammes per litre.								
	A ₁ .	A ₂ .	B ₂ .	C ₂₁ .	C ₂₂ .	D ₂₂ .	E.	F ₁₁ .	Control.
0	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
2	1.8	—	—	1.8	1.8	1.8	1.8	Trace	1.8
3	—	Trace	Trace	—	—	—	0.05	—	1.8
4	0.8	—	—	1.8	0.6	1.8	—	Nil	1.8
6	—	Nil	Nil	—	—	—	Trace	—	1.8

No nitrate was formed in any of the cultures, so it was clear that none of the eight organisms could oxidise nitrite into nitrate.

It will be observed that A₂, B₂, E and F₁₁ utilised the whole of the nitrite originally present in the medium. A₁ and C₂₂ appeared to assimilate it partially, while C₂₁ and D₂₂ did not absorb nitrite at all in the time under consideration.

It is well known that nitrate-forming bacteria can function only in a medium containing nitrite, but it is very curious that nitrite-forming organisms should absorb any nitrite that they themselves have produced from ammonium salts. It seems probable therefore that, although essentially nitrite formers, they also can use nitrite for their metabolism. Certain soil Actinomycoetes are capable of utilising nitrite when it is present in low concentrations.

With a view to ascertaining the effect of soil on the nitrifying powers of these

Nitrite Formation by Soil Bacteria.

bacteria, the production of nitrite was studied in sterilised soil treated with sulphate of ammonia and inoculated with each species.

For this purpose air-dried Barnfield soil receiving farmyard manure was employed. The soil was passed through a 2-mm. sieve and sterilised by heating in an air oven for 1 hour at 190° C. 300 gm. portions of this soil were moistened with 60 c.c. of an aqueous solution of 0.1 per cent. ammonium sulphate and were distributed into 2-litre conical flasks previously sterilised. The soil was inoculated with 1 c.c. of bacterial suspension. 20 gms. of soil were periodically withdrawn, shaken up with 100 c.c. of distilled water and the nitrite estimated in the extract by the Griess-Ilosva method.

The nitrite formed in each case is shown in Table IV.

Table IV.—Production of Nitrite from Ammonium Sulphate in Soil by Bacteria A₁, A₂, B₂, C₂₁, C₂₂, D₂₃, E and F₁₁. NO₂-nitrogen in milligrammes per kilo of soil.

Days.	A ₁ .	A ₂ .	B ₂ .	C ₂₁ .	C ₂₂ .	D ₂₃ .	E.	F ₁₁ .	Control.
2	1.0	Nil	Nil	Nil	Nil	Trace	Trace	Nil	Nil
3	1.7	„	„	Trace	Trace	0.6	0.6	Trace	„
4	—	—	—	0.7	—	0.6	0.6	„	„
5	2.0	Trace	Trace	—	0.25	—	—	0.8	„
6	1.7	„	„	1.3	Trace	1.3	1.3	1.7	„
8	0.9	0.6	Nil	Trace	Nil	1.9	1.7	1.7	„
9	—	—	—	„	—	1.9	1.3	—	„
10	—	—	—	„	—	Trace	1.3	—	„
11	0.4	0.5	Nil	0.4	Trace	„	1.3	1.0	„

It is seen that A₁, A₂, C₂₁, D₂₃, E and F₁₁ are definitely capable of oxidising ammonium sulphate into nitrite in soil. In none of the cultures was nitrate found.

Effect of Aeration.

Aeration has a favourable influence upon most soil organisms, and it may be assumed to be particularly beneficial to oxidising organisms. The influence of aeration on some of the strains isolated was therefore investigated.

A₂ and D₂₃ were selected for this purpose, since they showed such marked differences in their assimilation of nitrite. Two flasks each containing 100 c.c. of the mineral salt medium containing 0.1 per cent. ammonium sulphate were inoculated with A₂ and D₂₃ respectively. Air from which the carbon dioxide and nitrite had been removed was aspirated through the cultures at the rate of about 6 litres in 24 hours. The vessels were kept immersed in a thermo-

stat maintained at 23° C. and samples of the cultures for nitrite and bacterial determinations were taken by means of sterilised rubber tubing.

The results obtained are given in Table V.

Table V.—Effect of Aeration on the Oxidation of Ammonia to Nitrite by Strains A_2 and D_{33} . (Nitrite-nitrogen is expressed in milligrammes per litre and the bacterial numbers in millions per cubic centimetres.

Days.	A_2		D_{33}	
	$\text{NO}_2\text{-N.}$	Bacterial numbers.	$\text{NO}_2\text{-N.}$	Bacterial numbers.
0	Nil	31.0	Nil	44.0
2	0.62	48.6	0.5	35.7
5	1.0	78.6	1.5	25.7
7	0.5	Innumerable	1.4	26.8
8	0.25	„	1.25	32.0

Aeration evidently stimulated the production of nitrite especially during the first few days, since the amounts of nitrite given in Table V are greater than those given in Table II. The culture inoculated with A_2 showed a rapid increase in the bacterial numbers, whereas those in D_{33} tended to diminish. In A_2 the nitrite diminished with the age of the culture, but with D_{33} no such diminution was noticed; this was in accordance with the results already obtained (Table III), that while A_2 assimilated nitrite vigorously, D_{33} did not.

Experiments with Mixed Cultures.

Investigations were next carried out with mixed cultures in which the ammonia was produced from asparagin by the ammonifying bacterium “YB” isolated from soil in this laboratory.

The composition of the medium was :—

	Per cent.
K_2HPO_4	0.1
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.02
CaCl_2 ...	0.01
NaCl ...	0.01
FeCl_2 ...	Trace
Asparagin	0.05

The nitrogen equivalent of the asparagin added was 53 mgm. per litre, and the production of ammonia and nitrite was followed. The ammonia was estimated by Nessler's method ; and the results are given in Table VI.

Table VI.—Production of Ammonia and Nitrite from Asparagin by Mixed Bacterial Cultures. (Ammonia and nitrite expressed as nitrogen in milligrammes per litre.)

ains.	3 days.		6 days.	
	Nitrite.	Ammonia.	Nitrite.	Ammonia.
+ A ₁	0·5	40	0·85	50·0
A ₁	Trace	30	Nil	37·5
+ C ₁₁	0·4	12·5	0·4	20·0
C ₁₁	0·25	Trace	Nil	15·0
+ D ₁₁	0·8	37·5	0·75	37·5
D ₁₁	0·4	12·5	0·25	10·0
+ E	0·4	25	0·4	25·0
E	0·2	12·5	0·25	15·0
+ F ₁₁	0·5	25	0·5	25·0
F ₁₁	Trace	Trace	0·13	15·0

These experiments show that A₁, C₁₁, D₁₁, E and F₁₁ can all produce ammonia from asparagin though not to the same extent as YB. The nitrite formed from the ammonia that they themselves produce is, however, very low in comparison with the nitrite formed in association with YB. These nitrite organisms when alone can take an active part in breaking up the asparagin ; but during the process they possibly expend most of their energy and are able only to take a feeble part in oxidising this ammonia further into nitrite. When, on the other hand, they are associated with YB, the asparagin-ammonia transformation being mainly brought about by the latter bacteria, the nitrifiers are free to oxidise the ammonia produced into nitrite. Again there was no trace of nitrate in any of the cultures.

Description of Strains.

The characterisation of the strains according to the group number as arranged by the Society of American Bacteriologists is given as follows :—

Group number.	Characterisation.	
200	Endospores not produced	All.
10	Aerobic (strict)	All.
1	Gelatine liquefied	A ₂ , C ₂₂ , D ₃₃ , F ₁₁ .
2	Gelatine not liquefied	A ₁ , B ₂ , C ₂₁ , E.
0·4	No growth with dextrose*	All.
0·04	No growth with lactose	All.
0·004	No growth with saccharose	All.
0·0002	Nitrates reduced without gas	A ₁ , A ₂ , B ₂ , C ₂₁ , E, F ₁₁ .
0·0003	Nitrates not reduced	C ₂₂ , D ₃₃ .
0·00000	Non-chromogenic	All.
0·000003	Diastatic action on starch absent ..	All.
0·0000004	No growth with glycerine	All.

Total Group Number.

A ₁	212·4442034
A ₂	211·4442034
C ₂₁	212·4442034
C ₂₂	211·4443034
D ₃₃	211·4443034
E	212·4442034
F ₁₁	211·4442034

The characteristics of the organisms not used for finding the group number are as follows :—

Agar Slopes. 3 days old.—The growth of all the organisms was abundant ; echinulate, raised, smooth, glistening and opaque ; viscid and no odour ; medium unchanged.

Agar Stabs. 3 days old.—All uniform, filiform, medium unchanged.

Gelatine Stabs.—A₁, B₂, C₂₁, E, line of puncture filiform ; no liquefaction. A₂, C₂₂, D₃₃, F₁₁, surface growth, liquefaction.

Colonies on Thornton's Agar.—In all cases the growth is rapid, surface smooth, internal structure amorphous. There are minor differences in the character of the edge and the elevation, the edge in A₁, A₂, B₂, C₂₂, D₃₃ and E being entire, while that of C₂₁ and F₁₁ is undulating. The elevation of the colonies

* Besides the sugars mentioned here no growth was obtained with mannitol, maltose, laevulose and galactose.

of all the strains is convex, except in the case of A_3 , C_{23} and E, where it is flat.
Morphology and Staining.—These characters are given in Table VII.

Table VII.

strains.	Gram.	Shape.	Dimensions in μ .	Acid fast.	Moti
A_1	+	Rods	1.90×0.85	—	—
A_3	+	"	1.39×0.77	—	—
B_3	+	"	1.89×0.82	—	—
C_{11}	+	"	1.84×0.81	—	—
C_{23}	+	"	1.43×0.71	—	—
D_{13}	+	"	1.80×0.80	—	—
E	+	"	1.73×0.75	—	—
F_{11}	—	Coccus	0.93	—	—

It will be seen that some of these strains have a common group number and similar morphological characteristics, and therefore they may be classified into four groups :—

I.— A_1 , B_3 , C_{21} and E.

II.— A_3 .

III.— C_{23} and D_{33} .

IV.— F_{11} .

Reference to Table II shows that the oxidising power is not similar in what are probably the same strains ; but it must be remembered that in these experiments uniform inoculations were not made.

From the foregoing data it will be seen that in sharp contrast with Winogradsky's nitrite-forming bacteria the organisms described are capable of growing on nutrient agar and in the presence of sugar. They can produce nitrite within a wide range of p_H values varying from 4.8 to 7.3, whereas *Nitrosomonas* or *Nitrosococcus* can function only in a distinctly alkaline medium. Morphologically, too, they are different from *Nitrosomonas*, which first forms a zooglea and then breaks up into swarming, ellipsoidal, motile bacteria.

Summary.

Four species of non-spore-forming bacteria capable of oxidising ammonia into nitrite have been isolated from Rothamsted soil and all differ widely from *Nitrosomonas* or *Nitrosococcus*.

These organisms are able to carry out this reaction in artificial media as well as in soil, and some are able to assimilate nitrite.

Rapid growth takes place on nutrient agar, and the presence of 0.1 per cent. sucrose stimulates nitrite production.

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*Experiments on the Physiology and Genetics of the Smut Fungi.
Cultural Characters. Part II.—The Effect of Certain External
Conditions on their Segregation.*

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Introduction.

The researches, which are described under the above general heading, were begun in 1925. Their object was to find out if the inheritance of the characters in the smut fungi was similar to that in the higher plants and animals. The evidence presented, and to hand, was in the previous paper (1928) considered sufficient to justify the conclusion that, in their segregation, these fungi did obey the laws of Mendel. In that paper certain of the cultural characters were shown to be segregated in ratios of 2:2, 3:1 and 4:0 in those cells which were deduced to be the immediate progeny of the reduction divisions. The anomalous ratio of 3:1 was at that time the only difficulty, and this was, it seemed, probably due to the interaction of a number of Mendelian factors. Since that time segregation ratios of 2:1:1 (or 1:1:2) and 1:2:1 have been recognised.

It is thought that the conclusion that the segregation in these fungi is along Mendelian lines is fully justified by the experiments described in this paper. In the fungi generally, as Brierley has stated (1929), "local peculiarities" may be expected, but, so far, in the smut fungi I find typical haploid inheritance with one possible exception. If the factor gender (sex) only is considered, the evidence is overwhelmingly in favour of strict Mendelian segregation, both in that the factors are segregated in pairs and, as demonstrated in the present paper, that segregation under normal circumstances is haphazard. Also when the cultural characters are considered, the evidence is conclusive except that in one character this peculiar 3:1 ratio is found.

In recent years interest has grown considerably in fungal genetics, and the late Professor Kniep thoroughly reviewed the previous work in 1930. Also Link (1929) reviewed much of the earlier work in a discussion on the problem of sex and heterothallism in the lower types, when he put forward a new nomenclature. But in the present paper I propose to continue the use of the

term gender to describe the phenomena observed, without prejudicing the question whether the term sex may not be fully justified. In such use physiological specialisation is implied without any morphological differentiation.

A paper by Fleroff (1923) has only recently come to hand, and his results have in most respects been confirmed by me. Fleroff describes in *Ustilago avenae* (Pers.) Jensen the production of viable chlamydospores together with the completion in pure culture of the nuclear life cycle. Kniep (1919) and Sartoris (1924) demonstrated this completion in *Ustilago violacea* (Pers.) Fuck., and *Ustilago Heufleri*, Fuck., respectively. But I have been, with some exceptions, unable to obtain this result in *U. Kolleri* Wille (*U. laevis* Kell. et Sw.). Under certain conditions dark brown chlamydospore-like bodies are readily produced, and under other conditions apparent nuclear fusion, but true chlamydospores have not been obtained as a general rule. It is hoped that in time the problem of the production of viable chlamydospores, which is apparently bound up with the behaviour of the fusion nucleus, will be solved.

Kharbush (1928) showed that in *Ustilago maydis* Corda [*U. zeae* (Beckm.) Ung.] meiosis occurred in the first nuclear division after germination, and that at this division four chromosomes were present, while in the haploid nuclei two chromosomes were present. In *U. Kolleri* the number of chromosomes would appear to be greater: I have counted at least six in the first nuclear division after germination.

In *U. zeae* (Beckm.) Ung., Stakman *et alii* (1929) have described numerous changes, which, following Baur's definition, can be classed as mutations. They have shown that the frequency of these mutations can be correlated with an increased nitrogen content in the medium, an observation of considerable interest in itself and in view of the effect of an increased nitrogen content on segregation, described in this paper. In addition, they have shown that many, but not all, of their mutations are stable and that they arise from uninucleate cells. Whether or not these mutations are due to chromosomal aberrations or to genic changes, a cytological examination may be able to decide. In one case it would appear as though the capacity for mutation had been segregated at the reduction divisions (Stakman, 1929, see p. 10).

Methods.

It has long been known that the immature chlamydospore contains two nuclei which fuse when the spore reaches maturity. On germination a short tube, the promycelium, is produced and the single nucleus passes into it. Two nuclear divisions follow, giving rise to four nuclei, which are distributed

in a row along the promycelium. Cross walls then divide the promycelium into four segments, and each segment proceeds to form a succession of sporidia. Earlier workers used to call the first cell formed by each segment a sporidium (considered homologous with a basidiospore) and the remainder conidia. I have decided to keep the term sporidium to refer to a cell budded off from the promycelium, and the term conidia to refer to the cells budded off from the sporidia or their progeny. From the observation that the four nuclei of the promycelium are arranged in the segments in the order of their formation, it is clear that if segregation of gender has taken place in the first nuclear division of the promycelium, then the apical and second segments will form sporidia of one gender, while the sporidia from the basal segments, numbers 3 and 4, will be of the other gender (see Table I). Again, if segregation of gender takes place only in the second nuclear division in the promycelium, the first and second segments will differ in gender, and also the third and fourth segments. Supposing that gender were to be segregated only in the third nuclear division in the promycelium, then the first formed sporidium of each segment will differ in gender from the second and subsequently formed sporidia from the same segment. And, similarly, if segregation were to take place only in the fourth nuclear division, then cultures arising from the first formed sporidia would each contain the two genders, while the second formed sporidia would differ from the third and succeeding sporidia from the same segment. This type of analysis can be continued indefinitely, but it is usually only possible to follow the segregations in the nuclear divisions in the promycelium.

In practice the method of procedure has been to germinate under a number of particular conditions, chlamydospores with a known origin, and then to isolate, using the Dickinson method of isolation (1926), the sporidia from each segment of a promycelium as formed, and to continue the process as long as desired. These isolated cells form the starting point of the strains whose reactions have been studied. These strains are kept in sets of four strains, each set of four containing the first or second, etc., formed sporidia from a particular promycelium, and each strain arising from a particular segment of that promycelium.

These promycelia were produced from chlamydospores which, unless otherwise stated, were taken from plants, grown from seed in whose production all precautions had been observed to keep clear from smut spores. This seed was germinated under sterile conditions, and infected in the laboratory with the desired combination of strains of *Ustilago Kollerii* (1927, b), and then kept under sterile conditions until past the seedling stage. Once the plants were past the

Table I.

To show segregation and its results when it takes place in the first or the second or the third or the fourth nuclear division of the promycelium.

The letters A, B represent the members of a pair of characters.

—	Segregation solely in 1st division.	Segregation solely in 2nd division.
Segment of promycelium.	First and rest of sporidia will be—	First and rest of sporidia will be—
Apical segment	A B	A B A B
Second segment	A B	B A B A
Third segment	B OR A	A OR B OR B OR A
Basal segment	B A	B A A B

Segregation solely in 3rd division.

Segment of promycelium.	First formed sporidium will be—	Second and the rest will be—	OR	First formed sporidium will be—	Second and the rest will be—
Apical segment	A	B		B	A
Second segment	B	A		A	B
Third segment	A	B		B	A
Basal segment	B	A		B	A
<i>ETC.</i>					

Segregation solely in 4th division.

Segment of promycelium.	First formed sporidium will be—	Second formed sporidium will be—	Third and subsequent sporidia will be—
Apical segment	(AB)	A	B
Second segment	(AB)	B	A
Third segment	(AB)	A	B
Basal segment	(AB)	B	A

Alternatively.

Segment of promycelium.	First formed sporidium will be—	Second formed sporidium will be—	Third and subsequent sporidia will be—
Apical segment	(AB)	A	B
Second segment	(AB)	A	B
Third segment	(AB)	A	B
Basal segment	(AB)	A	B

ETC.

seedling stage, they were believed to be immune from infection. After this stage the plants were transferred to my own garden, and as soon as they produced smutted heads, these heads were bagged to prevent contamination from other sources. When these smutted heads were thought mature, they were gathered and allowed to dry, still in the bags. Each head as soon as ready was labelled and placed in a new envelope. Chlamydospores produced in this way and formed by the union of strains Z2/2 and CSOK52/4 were used in the detailed experiments on the segregation of gender; while in the experiments where the segregation of the cultural characters was tested, the chlamydospores were those due to the combination of strains CSOK52/1 and CSOK52/4.

Though no new methods have been employed in obtaining the results described in this paper, so far as is known, all the precautions necessary both in isolation and in cultural work have been taken. The method of testing the genders is that described in a previous paper (1927, *a*). In the determination of the cultural characters of the strains, many of the cultures made have been preserved, and five examinations of these preserved cultures made at intervals of about 5 months have given identical results. The standard medium used for determining the characters was maltose 0.536 per cent., urea 0.011 per cent., KH_2PO_4 0.5 per cent., MgSO_4 0.25 per cent., KCl 0.25 per cent. and 1.5 per cent. agar, with the H-ion concentration adjusted to p_{H} 5.5 after sterilisation, and a temperature during growth of 18° to 18.5° C.*

In the work described in this paper considerable use has been made of the character gender, and it may be as well to point out that a number of experiments have been made to try and find out if there exist in the strains used more than two genders (Bauch, 1923; Stakman, 1927). These experiments have been made on the type of fusion occurring when different strains meet, as well as on the influence of external conditions on the type of fusion. It is not proposed to describe in detail at present the results of these experiments, but to record in outline the following observations. There is a slight correlation between the environmental conditions and the type of fusion-hypha produced, and there is also a tendency for certain combinations to be more productive of fusion-hyphae than others, while in addition the fusion between the hyphae of certain strains is largely lethal (that is, the fusion-hyphae as a rule die almost as soon as they are formed). But in no case and in no way could any clear distinction always be made, and never was there any suggestion of the appearance of more than two genders.

* I wish to record my thanks to the Government Grant Committee, Royal Society, for the provision of incubators necessary for this work.

Experiments.

In the previous part (1928) it was shown that under certain conditions the segregation of gender and of the cultural characters took place in the nuclear divisions preceding sporidial formation in the promycelium. These conditions have been taken as standard, and the effect of different germination conditions on segregation were tested in one experiment on the cultural characters and in detail on the most reliable character, namely, gender.

A preliminary experiment was made to find out what number of promycelia ought to be tested to obtain a representative sample of the behaviour. For this experiment sporidia were isolated from a number of promycelia, produced under the standard conditions of germination. This was repeated six times, spreading over some 2 years in all. The arrangements of the genders in the promycelia were determined and from the figures it is possible (see Table I) to find out the number of promycelia in which segregation took place in the first nuclear division. In addition by determining the number of nuclear divisions in which A gender was segregated towards the apex of the promycelium, it is possible (see Table I) to find out whether either gender tended to be segregated towards one end of the promycelium rather than the other end. Table II shows the results obtained expressed as percentages. It will be

Table II.

Experiment to determine the reliability which can be placed on the results.

Germination conditions.—Temperature 18.5° C. H-ion concentration adjusted to p_H 6.0. Maltose concentration 0.0156 molar. Urea concentration 0.002 molar. Ratio of molecular concentrations of maltose and urea (C/N) = 8.

Month.	Number of promycelia whose gender distributions were determined.	Percentage of promycelia with segregation in 1st division.	Percentage of nuclear divisions in which segregation of A gender was apical.
August	36	44.4	51.7
October	23	51.7	47
February	50	48	48.6
April	29	48.2	51.6
September	32	50	50
Mean.....	34	48.5 $\pm$ 0.7	49.8 $\pm$ 0.6

noticed that the number of promycelia tested varied from 23–50, and that the percentage segregating in the first division was 48.5 with a probable error of $\pm$ 0.7.

A further preliminary experiment was then made to determine whether chlamydospores with different origins gave similar results to those given by the chlamydospores of the previous experiment. For this purpose three different kinds of chlamydospores were germinated under the standard conditions and the percentage segregation of gender in the first nuclear division determined, as well as the percentage of nuclear divisions in which A gender was segregated towards the apex of the promycelium. One source of the chlamydospores was a field at Seascale, Cumberland, where 75 per cent. of the plants were infected; while the other two "breeds" of chlamydospores were those formed by the combinations of the strains Z2/1-Z2/3 and CSOK52/1-CSOK52/4 produced in my own garden. The results (see Table III)

Table III.

Experiment to determine whether chlamydospores with different origins gave similar results.

Germination conditions.—The same as in the previous experiment (Table II).

Origin or "breed" of chlamydospores.	Number of promycelia whose gender distributions were determined.	Percentage of promycelia with segregation in 1st division.	Percentage of nuclear divisions in which segregation of A gender was apical.
Standard (Z2/2-CSOK52/4)	34	48.5 ± 0.7	49.8 ± 0.6
Seascale	36	27.7	46.8
Z2/1-Z2/3	24	25	52.3
CSOK52/1-CSOK52/4	35	45.7	51.4

suggest that under identical conditions of germination "breeds" of chlamydospores differ somewhat in the division in which segregation of gender occurs, but that there is no tendency for one gender to be segregated to one end rather than to the other end of the promycelium.

These preliminary experiments show that under the standard germination conditions,* 48 per cent. of segregation of gender in the first nuclear division is to be expected with chlamydospores of the "breed" Z2/2-CSOK52/4, and that a fair sample can be obtained when between 30 and 40 promycelia are tested. Further chlamydospores, produced by different combinations of

* The standard germination conditions were:—A medium containing 0.536 per cent. maltose, 0.011 per cent. urea, 0.5 per cent. KH_2PO_4 , 0.25 per cent. MgSO_4 , 25 per cent. KCl and 1.5 per cent. agar; a temperature of 18.5° C.; and an H-ion concentration adjusted to p_H 6.0.

strains, differ in the percentage of segregation of gender in the first nuclear division. In addition, neither gender tends to be segregated to one end rather than to the other end of the promycelium.

Before describing the observations made on the nuclear division in which segregation of the cultural characters and of gender occurred as affected by the environmental conditions, it is necessary to discuss some of the conclusions to which I have come after cultural work of some 3 to 4 years.

To determine the cultural behaviour of a particular strain, it is best to grow it for comparison with the remainder of the strains from the same promycelium together with two or three strains whose reactions are well known. It was found quite early that no comparison could be made on the basis of the general appearance; and that there were certain characters which appeared fairly constant and so could be relied upon. And it is the observations of the segregation ratios of some of these characters in the sets of four strains which will now be considered. It is frequently found that a slight change in the medium produces a marked effect on the amount of growth as measured by the diameter of the colony. This was well demonstrated in the case of CSOK52 (see Table IV). Here with the medium adjusted to p_H 5.4, strains 1

Table IV.

To show the variation in the amount of growth, as measured by the diameter in arbitrary units, of the four strains isolated from the promycelium CSOK52, when the H-ion concentration of the medium is slightly altered.

p_H .	Apical segment.	Second segment.	Third segment.	Fourth segment.
5.4	2	1	1	2
5.7	3	2	3	2
6.1	4	2	4	3

and 4 had an equal amount of growth, and about twice the amount of strains 2 and 3. With the same medium adjusted to p_H 5.7, the amount of growth was about half as much again in strain 1, while strain 2 had about twice the amount of growth as at p_H 5.4, and strain 3 showed three times the amount of growth, but strain 4 produced the same amount as at p_H 5.4. In the previous part (1928), the results of similar growth experiments were given, and these have been continued, but, from the observations just described, the amount of growth as measured by the diameter of the colony is evidently not a suitable character for the present purpose. Other characters, such as the type of

colony centre, have been found to vary less with slight changes of the medium, and when variation did occur, it affected all four strains in a similar manner, and no change from one type to another has ever been observed. It must be mentioned that colour is rather susceptible to temperature, an observation which Rodenhiser (1928) has made in *U. nuda*, etc.

In the majority of the sets of four strains it was fairly easy to determine the segregation ratios if the unit used was the particular character and not the general appearance. In the accompanying table (see Table V) examples have been given to show the type of result which was obtained. The first

Table V.

To show examples of the segregation of the cultural characters.

Set No.: 15.	Characters.	No. 1. Apical segment.	No. 2.	No. 3.	No. 4. Basal segment.
First formed sporidium	Gender	B	A	B	A
	Size of margin	Wide	Wide	Narrow	Narrow
	Colour	Brown	Brown	Cream	Cream
	Type of colony centre	Corrugated	Corrugated	Depressed	Depressed
	Surface	Moist	Moist	Dry	Dry
	Amount of growth at p_H 5.5	2	2	2	2

The cultures arising from the second formed sporidium were identical with those from the first formed sporidium.

Set No.: 21.	Characters.	No. 1. Apical segment.	No. 2.	No. 3.	No. 4. Basal segment.
First formed sporidium	Gender	B	A	A	B
	Size of margin	Wide	Narrow	Narrow	Wide
	Colour	Cream	Brown	Brown	Cream
	Type of colony centre	Depressed	Corrugated	Corrugated	Depressed
	Surface	Moist	Moist	Dry	Dry
	Amount of growth at p_H 5.5	2	1	1	2

The cultures arising from the second formed sporidium were identical with those from the first formed sporidium.

Table V—(continued).

Set No. : 4.	Characters.	No. 1. Apical segment.	No. 2.	No. 3.	No. 4. Basal segment.
First formed sporidium	Gender	B	A	B	A
	Colour	Yellow	Yellow	Yellow	Yellow
	Type of colony centre	Depressed	Corrugated	Depressed	Corrugated
	Surface	Moist	Moist	Dry	Dry
	Amount of growth at p_H 5.5	2	2	1	1

The cultures arising from the second formed sporidia were identical with those from the first formed sporidia.

Set No. : 32.	Characters.	No. 1. Apical segment.	No. 2.	No. 3.	No. 4. Basal segment.
First formed sporidium	Gender	B	A	B	A
	Size of margin	Wide	Wide	Narrow	Narrow
	Colour	Cream	Brown	Yellow	Yellow
	Type of colony centre	Corrugated	Corrugated	Depressed	Depressed
	Amount of growth at p_H 5.5	2	2	1	1
	Number of radial lines	—	—	Many	—

The cultures arising from the second formed sporidia were identical with those from the first formed sporidia except :—

	Number of radial lines	—	—	Few	—
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Set No. : 86.	Characters.	No. 1. Apical segment.	No. 2.	No. 3.	No. 4. Basal segment.
First formed sporidium	Gender	B	B	A	A
	Size of margin	Narrow	Wide	Narrow	Wide
	Colour	Cream	Cream	Brown	Brown
	Type of colony centre	Corrugated	Depressed	Intermediate	Intermediate
	Surface	Dry	Dry	Moist	Moist
	Amount of growth at p_H 5.5	1	2	1	3

The cultures from the second formed sporidia were the same as those from the first.

Table V—(continued).

Set No. : 33.	Characters.	No. 1. Apical segment.	No. 2.	No. 3.	No. 4. Basal segment.
First formed sporidium	Gender	B	B	A	A
	Size of margin	Narrow	Wide	Narrow	Wide
	Colour	Yellow	Yellow	Cream	Brown
	Type of colony centre	Corrugated	Corrugated	Depressed	Depressed
	Surface	Moist	Moist	Dry	Dry
	Amount of growth at p_H 5.5	1	2	1	2

The cultures arising from the second formed sporidia differ from those from the first formed sporidia in the following :—

	Colour	Yellow	Yellow	Brown	Cream
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Set No. : 23.	Characters.	No. 1. Apical segment.	No. 2.	No. 3.	No. 4. Basal segment.
First formed sporidium	Gender	A	B	A	B
	Size of margin	Narrow	Wide	Narrow	Wide
	Colour	Cream	Brown	Cream	Brown
	Type of colony centre	Depressed	Depressed	Depressed	Corrugated
	Amount of growth at p_H 5.5	2	2	2	2

five sets of four strains treated were given in the previous part (1928), but here six sets out of the total available have been picked out to illustrate the different types of segregation obtained.

In some cases, for example 15, the four strains were of two types only in every particular so far as has been tested. The one type had a wide margin, a brown colour towards the centre, with some corrugations towards the centre and a moist centre to the colony. The other type had a narrow margin, a cream colour, with radial lines starting from the centre called "depressed" and a dry centre to the colony. The two strains of each type were apparently identical, except as regards gender, while the amount of growth, i.e., diameter, was the same in all four strains. The second formed aporidial strains were identical with the first formed. Hence in all the characters except the amount of growth the segregation ratios appear to be 2 : 2. Since the two apical seg-

ments produced similar strains, and the basal segments had given rise to the other pair of strains, evidently the segregation had taken place in the first division. As the two apical segments differed in gender, the segregation of gender had taken place in the second division. Because segregation had taken place in different nuclear divisions, it is presumed that gender is independent of all the other characters except the amount of growth.

In 21 the strains were, in four characters, of two types ; the one with a wide margin, a cream colour, with the depressed type of colony centre and a large amount of growth. The other type had a narrow margin, a brown colour, with some corrugations in the centre of the colony and a small amount of growth. The gender of the former type was B. From the arrangement of the parent sporidia of these strains on the promycelium, all these characters had evidently been segregated in the second nuclear division in the promycelium. Similarly, the character dry or moist centre to the colony is deduced to have been segregated in the first nuclear division in the promycelium. Now since this character was segregated in a different nuclear division from the remainder of the characters, it must be presumed that it is independent of them.

In 4, excluding for the moment the character colour, it was found from the distribution of the strains on the promycelium, that the characters moist or dry surface of the colony and amount of growth had been both segregated in the first nuclear division in the promycelium, while the character type of colony centre had been segregated in the second nuclear division. The character type of colony centre is presumably independent of the amount of growth. In these three sets of four strains all the characters, except the amount of growth, have been segregated in ratios of 2 : 2. But in this set the colour of all four strains was the same, yellow, that is, a ratio of 4 : 0.

In 32 the width of margin, the type of colony centre and the amount of growth have all been segregated in a ratio of 2 : 2 in the first nuclear division. But three types of colour, cream, brown and yellow were present. One of the strains was cream, another was brown and the other two were yellow. The cream and brown strains were the product of one of the second nuclear divisions, and the two yellow strains were the product of the other. This gives a segregation ratio of 1 : 1 : 2, yellow being intermediate. Further, in this set of four strains, the second formed sporidial strain of the third segment of the promycelium differed from the first formed of that segment, in having only a few radial lines in contrast to many lines. Consequently, since gender was segregated in the second nuclear division, the segregation had been spread over three consecutive nuclear divisions. In addition it must be presumed

Table VI.

To point out the sets of four strains in which the segregation of the following pairs of characters takes place in different nuclear divisions.

Character pairs.	Sets of four.
Gender and size of margin	15, 86, 33
" colour	15, 33
" type of colony centre	15, 86
" surface	15, 21, 4
" amount of growth at p_H 5.5	4, 86, 33
" number of radial lines	32
Size of margin and colour	32, 86
" type of colony centre	33
" surface	21, 86, 33
" amount of growth at p_H 5.5	—
" number of radial lines	32
Colour and type of colony centre	32, 86, 33
" surface	21, 33
" amount of growth at p_H 5.5	32, 86, 33
" number of radial lines	32
Type of colony centre and surface	21, 4, 86
" " amount of growth at p_H 5.5	4, 33
" " number of radial lines	32
Surface and amount of growth at p_H 5.5	21, 86, 33
" number of radial lines	—
Amount of growth at p_H 5.5 and number of radial lines	32

that gender and the number of radial lines are both independent of each other, and of the characters width of margin, colour, type of colony centre and amount of growth.

In 86 the gender, the colour and the moistness of the colony surface were segregated in the first nuclear division, while the size of margin and the amount of growth were segregated in the second nuclear division. Therefore the character colour is independent of the characters size of margin and the amount of growth of the colony. But the strains showed three types of colony centre; the two extreme types were the product of one of the second nuclear divisions, while the other two strains were alike and intermediate and the product of the other second nuclear division; hence a ratio of 1 : 1 : 2. Similar to this segregation ratio of 1 : 1 : 2 (or 2 : 1 : 1) are certain cases where the two intermediate types are not the product of the same second nuclear division. Such a ratio, which has only been found in the character type of colony centre, is called a 1 : 2 : 1 ratio.

In 33 there is a set of four strains which helps in the interpretation of the ratios 2 : 1 : 1 (or 1 : 1 : 2). Colour in this case is also of three types. The four strains from the apical and the second segments were yellow in colour; the third and basal segments, however, produced first a cream and a brown

strain respectively, and secondly *vice versa*. Colour has been segregated in this half of the promycelium in the third nuclear division; and in the third segment the brown character remained in the promycelium, but in the fourth segment the cream character remained. Such a case shows that the ratio of 2 : 1 : 1 is regularly produced. Strains 3*b* and 4*a* as well as 3*a* and 4*b** differ only in the size of margin and the amount of growth. Since the type of colony centre was segregated in a different division from the margin size and the amount of growth, it is independent of them. The only two pairs of characters of those described, which have not been shown to be independent by their segregation in a different nuclear division, are the size of margin and the amount of growth, and the type of colony surface and the number of radial lines. The independence of these characters has been demonstrated in other sets of four strains.

The characters of the set of four strains Z3 are repeated and amplified from the first part of this paper (1928), because it is one of the best examples of what was then described as a ratio of 3 : 1, in the segregation of the type of colony centre. Strains numbers 1 and 2 were practically identical in this character, while strain number 3 was almost the same, and strain number 4 was markedly different. The interpretation given to such a ratio will be discussed later; in the meantime it may be stated that while the similarity between strains 1, 2 and 3 is close, and is always obtained on repetition, there is rather more difference between 1 or 2 and 3 than between 1 and 2.

From the above description it would appear that all the characters mentioned are independent of each other, and that segregation in a promycelium may take place only at the first nuclear division, or only at the second nuclear division, or it may take place in both these divisions. In addition, while no promycelium has been obtained in which segregation only took place in the third nuclear division, some examples have been obtained in which segregation had taken place in the first, the second, and the third divisions, as well as cases of segregation only in the first and the third, and only in the second and the third divisions. Segregation then, it seems, is not confined to one or to any particular nuclear division. In the previous part of this paper, the statement was made that no segregation had been found beyond the first two nuclear divisions in the promycelium. This was strictly limited to the standard conditions, for the results on third division segregation, although suspected at that time, had not been confirmed. Segregation in the third nuclear division has been found in all the characters except gender and the amount of

* Suffices *a* and *b* denote first or second formed sporidium respectively.

growth, but not in every character has it been found in different sets of four strains. The chief feature of segregation in the third nuclear division is that the differences between the strains in culture are more clear cut and precise than in many of the cases of segregation in other nuclear divisions.

In the set of experiments on the segregation of the cultural characters, all the chlamydospores came from the same inflorescence of a plant which had been infected with strains numbers CSOK52/1 and CSOK52/4. This plant was grown under sterile conditions until past the seedling stage ; in consequence these chlamydospores were almost certainly the product of the combination CSOK52/1-4. As an extra safeguard, all four strains of a set of four have been grown together with two or more strains whose characters were well known ; and I think that a certain amount of comparison can with safety be made between a pair of characters in one set of four and that in another. That is, that within limits the character cream is the same character in all sets of four strains.

For the purpose of testing the effect of the germination conditions on the segregation of the cultural characters, the three most satisfactory characters, the size of margin, the colour and the type of colony centre were selected. The first and second formed sporidia were isolated from promycelia which had been produced in the one case under the standard germination conditions, and in the other case with an increased concentration of the nitrogen source in the medium. Under the standard conditions the progeny of 35 different promycelia were available, and the characters of the strains were determined under the appropriate standard conditions (see methods section). The progeny of 28 different promycelia were available in the second case, and the reaction of the strains were likewise determined (see Table VII). It will be apparent from this table that, under the standard conditions, the percentage of segregation in the first division varied in the different characters, the maximum percentage being found in the segregation of gender and the size of margin, with a lower percentage in the type of colony centre, and a still lower percentage in the case of colour. While statistical proof is lacking, it is considered that these differences between the characters' segregation are real, and that characters do differ in their percentage of segregation in the first division. Further, the effect of increasing the nitrogen source concentration in the medium has been to lower, more or less uniformly, the percentage of segregation in the first division. Since the percentage of nuclear divisions in which one character of each pair was segregated towards the apex of the promycelium was approximately 50 in each of the characters, there has been haphazard distribution of

Table VII.

Experiment to show the effect of increasing the concentration of the nitrogen source in the germination medium on the nuclear division in which segregation of certain cultural characters takes place, and the distribution of those characters in the strains arising from the promycelia.

Concentration of nitrogen source.	C/N.	Gender.			Size of margin.		
		Number of promycelia tested.	Percentage segregation in first division.	Percentage nuclear divisions A gender terminal.	Number of promycelia tested.	Percentage segregation in first division.	Percentage nuclear divisions wide terminal.
Mol.							
0.002	8	35	45	51	34*	47	50
0.0625	$\frac{1}{4}$	28	39	47	28	32	51

Concentration of nitrogen source.	C/N.	Colour.			Type of colony centre.		
		Number of promycelia tested.	Percentage segregation in first division.	Percentage nuclear divisions brown terminal.	Number of promycelia tested.	Percentage segregation in first division.	Percentage nuclear divisions corrugated terminal.
Mol.							
0.002	8	31†	23	47	29†	38	53
0.0625	$\frac{1}{4}$	23†	4	45	19†	16	48

* One case undetermined through loss of parent culture.

† Due to subtraction of cases of 3 : 1 and 4 : 0 ratios.

Percentage taken to nearest whole numbers.

the character pairs in the promycelium. These observations would not prove that the pairs of characters were independent of each other, and in the next table (see Table VIII) the percentage of the number of times is shown, in which two members from two different pairs of characters have been segregated to the same strain. This is further evidence that the pairs of characters considered are independent of each other.

The frequency of the occurrence of the different segregation ratios is shown in the next table (see Table IX). It will be noticed that cases of 2 : 2 ratios only have been found in the character size of margin, and this has previously been demonstrated to be the case in gender (1928). Further 2 : 2, 2 : 1 : 1

Table VIII.

To show the correlation between certain characters under the two germination conditions.

Concentration of nitrogen source.	C/N.	A gender and narrow margin.	A gender and brown.	A gender and corrugated.	Narrow margin and brown.	Narrow margin and corrugated.	Brown and corrugated.
Mol. 0.002	8	45	52	46	52	50	45
0.0625	$\frac{1}{4}$	53	49	51	54	51	50

Table IX.

To show the frequency of occurrence of segregation ratios for the characters gender, size of margin, colour and type of colony centre, under the two germination conditions.

Concentration of nitrogen source.	C/N.	Total number of promycelia available.	Number of promycelia showing gender segregation ratios of—						Number of promycelia with segregation in third division.
			First division 2:2	Second division 2:2	2:1:1	1:2:1	3:1	4:0	
Mol. 0.002	8	35	16	19	—	—	—	—	—
0.0625	$\frac{1}{4}$	28	11	17	—	—	—	—	—

Concentration of nitrogen source.	C/N.	Total number of promycelia available.	Number of promycelia showing size of margin segregation ratios of—						Number of promycelia with segregation in third division.
			First division 2:2	Second division 2:2	2:1:1	1:2:1	3:1	4:0	
Mol. 0.002	8	34*	16	18	—	—	—	—	—
0.0625	$\frac{1}{4}$	28	9	19	—	—	—	—	1

* One case undetermined through loss of parent culture.

Table IX—(continued).

Concentration of nitrogen source.	C/N.	Total number of promycelia available.	Number of promycelia showing colour segregation ratios of—						Number of promycelia with segregation in third division.
			First division 2:2	Second division 2:2	2:1:1	1:2:1	3:1	4:0	
Mol. 0.002	8	35	8	15	8	—	—	4	—
0.0625	$\frac{1}{4}$	28	1†	18†	4†	—	—	5†	4

Concentration of nitrogen source.	C/N.	Total number of promycelia available.	Number of promycelia showing type of colony centre segregation ratios of—						Number of promycelia with segregation in third division.
			First division 2:2	Second division 2:2	2:1:1	1:2:1	3:1	4:0	
Mol. 0.002	8	35	11	8	8	2	3	3	—
0.0625	$\frac{1}{4}$	28	3	6†	10†	1†	2†	5†	2

† Includes cases where segregation of some characters occurred in the third nuclear division.

and 4:0 ratios have been found in the character colour, while it is only in the character type of colony centre that ratios of 1:2:1 and 3:1 have been found. In the discussion section, these results are interpreted on the basis that the character margin size is the expression of one pair of Mendelian factors, colour the expression of two pairs of additive linked Mendelian factors, and that the character type of colony centre is the expression of more than two pairs of factors. It is to be regretted that the numbers are insufficient to indicate whether the frequency of any ratio is affected by the external conditions.

In Tables VII and VIII, the distribution of the members of the character pairs was haphazard in the promycelium, and irrespective of each other. But it must be noted that to obtain these figures the strains intermediate in these characters were neglected. Now if these conclusions are correct, and if it also applies for the two pairs of factors in the case of colour, one would expect that the number of nuclear divisions, where brown and cream were segregated, would be the same as where the intermediate colour resulted. In Table X are given the proportions of these two types of segregation where segregation has taken place in the second nuclear division. Similarly the same proportions

Table X.

To show the proportion of nuclear divisions where brown and cream were segregated to those where yellow was segregated, in both cases only those in the second division. Similarly for the type of colony centre character, (a) excluding cases of 3 : 1 ratios, and (b) including these cases as 2 : 1 : 1 ratios.

Concentration nitrogen source.	C/N.	Ratio of divisions where brown and cream resulted to those where yellow resulted.		Ratio of divisions where depressed and corrugated resulted to those where intermediates resulted.	
		Total No.	Ratio.	Total No.	Ratio.
Mol. 0.002	8	54	2.4 : 1	(a) 42 (b) 48	1.6 : 1 1.5 : 1
0.0625	$\frac{1}{4}$	54	2.9 : 1	(a) 44 (b) 50	1.1 : 1 1 : 1

have been extracted for the character type of colony centre. These proportions show that in the case of colour the distribution was not haphazard, and that there is a connection between the two pairs of colour factors. But in the case of type of colony centre, the proportions obtained are not sufficient to justify such a conclusion, and, as discussed later, the occurrence of a ratio of 1 : 2 : 1 is probably an indication that there is not a connection between some, at any rate, of the factor pairs, whose expression is seen in the type of colony centre. Further, if the connection, as suggested by the proportions, between the colour factors is affected by the increase of the nitrogen source concentration, the proportions should be different for the two germination conditions. As these numbers are based on 54 different nuclear divisions in each of the two conditions, they are perhaps sufficient to warrant a conclusion, and a precedent for such an effect lies in the results of Plough (1917, 1921), who showed that temperature altered certain linkages in the female in *Drosophila*.

Returning now to Table IX, it will be apparent that only with an increased nitrogen source concentration in the medium was any segregation in the third division obtained; and it is interesting to note that the number of times segregation occurred for the three characters follows roughly the percentage of segregation in the first division, although the numbers are too small to be significant. It would appear, however, that the postponement of segregation

to the third division is due to the increase of the concentration of the nitrogen source in the medium. The nature of this effect on the segregation as a whole is of considerable interest; whether it is merely a postponement of the change from the diploid to the haploid state for a further nuclear division, or whether there is in addition an extension of the change so that, instead of taking place at one nuclear division, it is spread over a number of nuclear divisions. To ascertain the nature of the effect a table (see Table XI) has been compiled giving the number of promycelia, which in the characters investigated showed segregation only in one division, in two divisions and in three divisions, arranged according to the conditions of germination. The results suggest that the effect is extension rather than simply postponement from one nuclear division to another.

Table XI.

To show the number of promycelia with segregation of the four characters gender, size of margin, colour and type of colony centre, in one, two and three nuclear divisions under the two germination conditions.

Concentration of nitrogen source.	C/N.	Number of promycelia available.	Number of promycelia with segregation in—			Proportions.
			One division.	Two divisions.	Three divisions.	
Mol. 0.002	8	34	8	26	—	1 : 3.2 : 0
0.0625	$\frac{1}{4}$	28	2	20	6	1 : 10 : 3

The experiment on the segregation of the cultural characters involved a considerable amount of work, and it has only been possible to make an examination in detail of the effect of the germination conditions on segregation with the character gender. It was decided to test the effect of varying the concentrations of the nitrogen and the carbohydrate sources in the medium, as well as the concentration of the medium itself. In addition the effect of the temperature and of the H-ion concentration was also tested. In each experiment, except two, the number of promycelia used was over 30. The results are given in the following five tables.

Taking the first table (see Table XII) as an example, the effect of altering the concentration of the nitrogen source in the medium is shown. The concentration varied from 0.002 to 0.0625 to 0.125 molar, and the percentage

Table XII.

Experiment to show the effect of increasing the concentration of the nitrogen source in the medium.

Germination conditions.—Temperature 18.5° C. H-ion concentration adjusted to p_H 6.0. Maltose concentration 0.0156 molar.

Concentration of nitrogen source.	C/N.	Number of promycelia whose gender distributions were determined.	Percentage of promycelia with segregation in first division.	Percentage of nuclear divisions in which segregation of A gender was apical.
Mol.				
0.002	8	34	48.5 ± 0.7	49.8 ± 0.6
0.0625	1	32	40.6	45.4
0.125	1	49	30.6	49.3

of segregation in the first division was 48, 40.6 and 30.6 respectively. While complete statistical proof is lacking, it seems that the effect of increasing the concentration of the nitrogen source in the medium is to postpone the segregation of gender more and more from the first to the second nuclear division in the promycelium, thus confirming the results of the previous experiment (see Table VII). A similar effect is seen in the next table (see Table XIII), where the factor altered was the concentration of the carbohydrate source in the medium.

Table XIII.

Experiment to show the effect of increasing the concentration of the carbohydrate source in the medium.

Germination conditions.—Temperature 18.5° C. H-ion concentration adjusted to p_H 6.0. Urea concentration 0.002 molar.

Concentration of maltose.	C/N.	Number of promycelia whose gender distributions were determined.	Percentage of promycelia with segregation in first division.	Percentage of nuclear divisions in which segregation of A gender was apical.
Mol.				
0.0156	8	34	48.5 ± 0.7	49.8 ± 0.6
0.0625	32	45	44.4	48.9
0.125	64	42	35.7	49.2

In the case of the external factors, viz., concentration of the medium itself (see Table XIV), H-ion concentration (see Table XV) and temperature (see

Table XVI), the results suggest that there is an optimum effect on the nuclear division in which gender is segregated. But because for the latter two factors one test each was made with a number of promycelia less than 30, the results are not so conclusive as those obtained with the former pair of factors. Probably an optimum point would also have been found if a different range of concentrations of the nitrogen and carbohydrate sources had been used.

Table XIV.

Experiment to determine the effect of varying the concentration of the medium.

Germination conditions.—Temperature 18.5° C. H-ion concentration adjusted to p_H 6.0.

C/N, molar concentration.	Concentration of maltose.	Number of promycelia whose gender distributions were determined.	Percentage of promycelia with segregation in first division.	Percentage of nuclear divisions in which segregation of A gender was apical.
8	Mol. 0.0039	25	32	56.8
8	0.0156	34	48.5 ± 0.7	49.8 ± 0.6
8	0.125	45	31	50

Table XV.

Experiment to determine the effect of varying the H-ion concentration of the medium.

Germination conditions.—Temperature 18.5° C. Maltose concentration 0.0156 molar. C/N 8.

H-ion concentration.	Number of promycelia whose gender distributions were determined.	Percentage of promycelia with segregation in first division.	Percentage of nuclear divisions in which segregation of A gender was apical.
p_H			
5.1	35	42.9	50.9
6.0	34	48.5 ± 0.7	49.8 ± 0.6
7.0	28	21.4	47.9

Table XVI.

Experiment to determine the effect of varying the temperature of germination.

Germination conditions.—H-ion concentration adjusted to p_H 6.0. Maltose concentration 0.0156 molar. C/N 8.

Temperature.	Number of promycelia whose gender distributions were determined.	Percentage of promycelia with segregation in first division.	Percentage of nuclear divisions in which segregation of A gender was apical.
° C.			
12	32	34.3	45.2
18.5	34	48.5 $\pm$ 0.7	49.8 $\pm$ 0.6
24.5	22	40.9	54.5

It is worthy of notice that in every experiment the distribution of the genders in the promycelium is in no way correlated with either end of the promycelium, in fact the distribution is haphazard, thus confirming the results shown in Table VII.

In these experiments on segregation, no conclusive evidence was obtained that the segregation of gender could take place in the third nuclear division. But it is of interest to note (see Table XVII) that, in the total number, 663,

Table XVII.

Total number of promycelia in which the distribution of gender has been determined in these experiments.....	= 663
Number of promycelia in which the ratio of genders was 1 : 1	= 648

of promycelia tested in the experiments previously described, some 15 of these have shown a ratio of 3 : 1 or 4 : 0 of gender. This may well have been due to faulty isolation—a personal error which is bound to occur where such large numbers are concerned—but it is also possible that these 15 or some of them may have been due to the segregation of gender in the third nuclear division. This latter suggestion is rendered the more likely since some five occurred when the effect of adjusting the H-ion concentration to p_H 7.0 was tested. Since the percentage of segregation in the first division was 21.4, it is in this experiment that the postponement of the segregation of gender to the third division is to be expected.

Discussion.

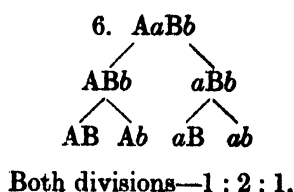
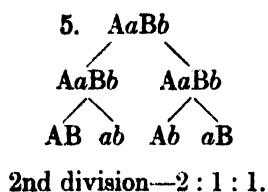
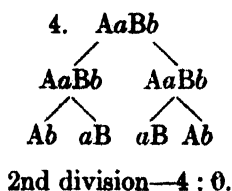
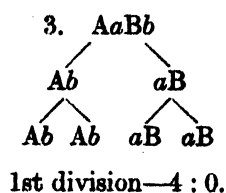
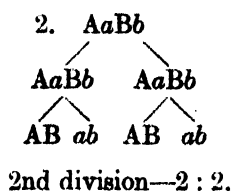
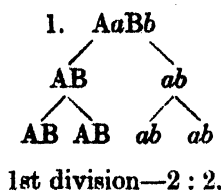
Although full statistical proof is lacking in the experiments with gender, I think that the size of sample having been over 30 warrants accuracy in the results. As regards the cultural characters, the observations made are not so reliable because there is more room for personal error; since repeated examinations, made during the last 2 years both by myself and by independent observers, have given identical results, these observations are considered sufficient to justify any conclusions which their numbers will warrant.

From the more recent literature (Brierley, 1929; Kniep, 1929) it would appear that evidence is in favour of the occurrence of normal Mendelian inheritance in the fungi. But Harder's work (1927) has shown that in some basidiomycetes, the cytoplasm has an influence, and this is perhaps supported by the results on "diploidisation" reported by Buller (1930). Although the possibility must be kept in mind of there being some characters in the smuts (Dickinson, 1927 *a*, 1928) on whose expression the influence of the cytoplasm may be demonstrated, it is considered that the nucleus only is responsible for the expression of the characters described in this paper.

By deduction from Mendel's principles, a segregation ratio of 1 : 1 is to be expected in haploid organisms, and this ratio has been found by von Wettstein (1924) in two mosses, and by Allen (1924-25) in a liverwort, as well as by later workers. The same ratio for gender was demonstrated by Kniep (1919) in *Ustilago violacea*, and in the previous part of this paper for *U. Kolleri*. Dodge (1930) has obtained a similar result for some characters in *Monilia sitophila*. In sex, a segregation of 1 : 1 in the units concerned is not apparently affected by the relative value and sign of the conjugants (Hartmann, 1925; Vandendries, 1929; Blakeslee, etc., 1927). And this complication has not as yet appeared in gender in *U. Kolleri*, as shown in the summary description of experiments and results, given in the methods section, combined with the figures in Table XVII.

A segregation ratio of 2 : 2 is at times found in all the characters described in this paper (see Tables V and IX), while their independence is shown by their segregation in different nuclear divisions. The characters gender and size of margin are always segregated in this ratio, but the two characters, colour and type of colony centre, at times are segregated in other ratios. For colour the observations point to the presence of two additive linked pairs of Mendelian factors as responsible for its expression. The occurrence of ratios of 1 : 2 : 1 and 4 : 0 indicates the presence of more than one pair of factors.

That with two linked pairs of factors the results could be obtained providing an additive effect, is seen by the following segregation schemes, in which are inducted the nuclear division or divisions at which segregation occurs :—



If it be assumed that with two colour factors (AB) a brown colour is obtained, and with either of them (Ab or aB) yellow, and with neither of them (ab) a cream colour, then the first five schemes give the ratios found. With no connection between the two factor pairs, there will be an equal number of schemes 1 and 3, and of schemes 2, 4 and 5. The figures given in Table IX, though small, suggest that this is not followed. In addition the 6th scheme, in which the two pairs of factors are segregated in different nuclear divisions, should be found unless the two factor pairs are connected or show linkage. But in all the promycelia investigated no case of a ratio of 1 : 2 : 1 in colour has been found. The amount of linkage, as well as its presence, can be seen by comparing the number of nuclear divisions in which the two colour factors are segregated together and to different daughter nuclei. The proportions, given in Table X, indicate that the two colour factors are segregated about $2\frac{1}{2}$ times more frequently together than separately. If the two pairs of factors were not linked, the proportions should be approximately equal, and as there are 54 nuclear divisions on which to base this result, it is considered reliable. Further only three types of colour have been found in a promycelium. If the effect were not additive then it should be possible not only to distinguish four types, but also to distinguish between a first and a second division 4 : 0 ratio (schemes 3 and 4).

For the type of colony centre, because there are found 1 : 2 : 1 and 3 : 1 ratios, it would seem probable that some of the pairs of factors are not linked, and that there are more than two of them. That the evidence does not justify

the suggestion of linkage is shown by the test of the distribution of the factor pairs (see Table X). A ratio of 3 : 1 is difficult to interpret unless one suggests that certain factors nullify the effect of others, but it is believed best to consider that these 3 : 1 ratios are due to the presence of multiple factors which interact on one another.

Although the effect of altering the external conditions on the frequency of occurrence of the different segregation ratios has not been demonstrated owing to the few promycelia treated, yet indirectly an effect can be deduced. As already concluded, there is linkage between the colour factors, while the proportions in Table X suggest that by increasing the nitrogen source content from 0.002 molar to 0.0625 molar, the linkage between the two pairs of colour factors is increased. And theoretically an increased linkage will result in a decreased number of promycelia with 2 : 1 : 1 and 4 : 0 ratios.

Coming to the haphazard distribution of the members of the character pairs, this was demonstrated by Kniep (1926) for *Ustilago violacea*, and in the previous part of this paper in *U. Kollerii*, and with *Coprinus* by Hanna (1925) and Newton (1926). In the present experiments the five environmental factors have no influence at all on the distribution of the genders in the promycelium; yet it is by no means improbable that there is one external factor at least (unpublished observations), which will influence the distribution of the chromosomes at meiosis; but these five factors are those, which, in our present state of knowledge, would appear to be the most likely to vary in and around a cell undergoing meiosis. Also (see Table VII), the distribution of the members, when the segregation ratio is 2 : 2, of the pairs of cultural characters in the promycelium, is haphazard, just as it is in gender. Further, Table VIII demonstrates that the distribution of a member of one pair of any of the characters used, is independent of that of a member of any other pair.

I think the evidence presented is sufficient to demonstrate the segregation of the cultural characters in "paired elements" or allelomorphs. In addition, the segregation of any pair of allelomorphs, excepting the cases of linkage, is independent of that of any other pair. These two rules of segregation comprise Mendel's laws, and consequently the segregation of these characters in the smut fungi is according to the principles of Mendelian inheritance.

Passing now to the nuclear division in which segregation occurs, Allen (1924-25) found that normally in a tetrad of spores of the liverwort *Sphaerocarpos Donnellii* only two types were present, but that occasionally four types appeared. He attributed this result to the occasional disjunction of chromosome pairs in different nuclear divisions. Similar results have been obtained

in insects by Wenrich (1916) and Carothers (1921), and in *Coprinus* by Newton (1926). Such demonstrations of disjunction and segregation in two consecutive nuclear divisions have been added to by the demonstration in these experiments of segregation in three consecutive and two non-consecutive divisions; these experiments show as well that the nuclear division in which segregation occurs is controlled in part by the surrounding medium. Since meiosis in *Ustilago Kollerii* is taking place in one cell in contact with the external environment, and not, as in *Coprinus* for example, immersed in the tissues of the organism, the effect is probably more easily demonstrated in the former than in the latter type.

The evidence indicates that the effect of the external medium is one of extending the process of segregation from one to two or three consecutive divisions. In addition, the independent pairs of factors are shown to differ in the percentage numbers of nuclear divisions in which their segregation occurs. This being the case, it would appear possible to obtain a promycelium where segregation of each of the independent pairs of factors had occurred in a different nuclear division. Further, as the external environment controls in part the onset of meiosis, and as segregation may take place in two non-consecutive divisions, it would appear possible that certain factors might only be segregated under certain conditions. This might provide an explanation of some cases of somatic segregation (Andersson, 1923), as well as suggesting that the external conditions of the cell are in part responsible for non-disjunction and the consequent types of polyploidy.

In conclusion, it is thought that these experiments indicate the importance of the meiotic process as a whole, that its onset and duration as measured in nuclear divisions is controlled in part by certain external conditions, that different chromosomes pairs vary in their reaction to these conditions, and that these particular external conditions have no effect on the distribution of the disjoining chromosomes.

Summary.

(1) Seven pairs of independent characters, shown by *Ustilago Kollerii*, Wille, in pure culture, are demonstrated to be segregated at times in a 2:2 ratio in the promycelium of the germinating chlamydospores.

(2) Of these seven pairs of characters, two—gender and margin size—are segregated always in a 2:2 ratio.

(3) Another character—colour of the colony—is segregated at times in 2:1:1 and 4:0 ratios.

(4) A fourth character—type of colony centre—is segregated at times in 2:1:1, 1:2:1, 3:1 and 4:0 ratios.

(5) The character pairs gender and margin size are deduced to be the expression of one pair of Mendelian factors.

(6) Colour is deduced to be the expression of two additive linked pairs of Mendelian factors.

(7) Type of colony centre is deduced to be the expression of a number of pairs of Mendelian factors.

(8) It is possible that the linkage between the two colour factor pairs is altered by a change in the nitrogen source concentration in the external medium.

(9) The distribution of the members of all seven pairs of segregating characters is haphazard, and is not affected by a change in the nitrogen source concentration in the external medium.

(10) Four of the seven pairs of characters differ in the percentage number of times they are segregated in a particular nuclear division.

(11) This percentage segregation is altered by changing the nitrogen source concentration in the external medium.

(12) The effect of increasing the nitrogen source concentration is to extend the process of segregation from one nuclear division to two or to three divisions.

(13) Segregation may, however, take place in two non-consecutive divisions.

(14) The percentage segregation of gender is altered by four other external factors in the medium.

(15) These external factors do not alter the haphazard segregation of gender.

(16) The results show that the onset and the duration—as measured in nuclear divisions—of the process of meiosis is in part controlled by the external environment of the cell.

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The Chromosome Constitution of the Pomoideæ.

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(Communicated by Sir Daniel Hall, F.R.S.—Received February 21, 1931.)

[PLATE 17.]

1. *Introduction.*

Previous investigations on cultivated varieties of apples have shown that the apparent basic chromosome number is 17, but chromosome pairing, morphology and breeding results indicate that the 34 chromosomes form a more complex constitution than that of an ordinary diploid.

In a recent paper (Darlington and Moffett, 1930) it was concluded that in the cultivated apples the set of 34 chromosomes in the diploid belong to seven types, three of which are represented six times and four of them are represented four times. Thus the original ancestor of the apple would have had a basic number of 7, and the present number of 17 is a secondary basic number. The derived series of polyploids with chromosome numbers 34, 51 and 68 are, therefore, secondary polyploids.

Since a change in chromosome balance is by analogy with other cases usually accompanied by a distinct change in the morphological characters of the plant concerned, it seemed possible that the Pomoideæ as a group might owe their special morphological characters to the state of secondary balance in the chromosome constitution. On this basis it was predicted that the secondary-balanced basic number of 17 would be found throughout the whole group, although other workers have reported different chromosome numbers in the group.

The investigations which are reported upon in the present paper were undertaken to see how far this prediction was in accordance with the facts.

2. *Methods and Material.*

Root tip preparations were studied in as many groups as possible, but owing to the common practice of propagating species of the Pomoideæ asexually by grafting, it was necessary to obtain seedlings of some species examined.

Pollen mother-cells were fixed in March, April and the early part of May. Medium Flemming and a modification of La Cour's 2BE (1931) were used as fixing solutions, and Newton's Gentian violet as a stain. The anthers were removed from the buds and fixed separately. Both fixatives gave good but somewhat variable results with the anthers.

Root tips were cut at 6 μ and anthers at 14 μ .

Most of the material for pollen mother-cell preparations was obtained from the Royal Botanic Gardens, Kew, by the kind permission of the Director.

I was able to examine root-tip material of one variety of cultivated pear, "Fertility," through the kindness of the Director of the East Malling Research Station, who supplied a plant of this variety on its own roots.

3. *The Classification of the Pomoideæ.*

Focke's system of classification in Engler and Prantl (1894) was followed as being the most convenient for these investigations, except as regards *Mespilus* and *Crataegus*, which have been considered as separate genera instead of being grouped together under *Mespilus*.

Genera of Pomoideæ.....Engler and Prantl.

1. *Cotoneaster*, Medic.

Section i. *Eucotoneaster*, Focke.

ii. *Pyracantha*, Röm.

2. (*Nagelia*, Lindl.).

3. *Osteomeles*, Lindl.

4. *Cydonia*, Tourn.Section i. *Eucydonia*, Focke.ii. *Chaenomeles*, Lindl.5. (*Docynia*, Decsne.)6. *Pyrus*, Tourn.Sub-genus i. *Pirophorus*, Medic.ii. *Malus*, Tourn.iii. *Hahnia*, Medic.iv. *Sorbus*, L.v. *Aronia*, Pers.7. *Rhapiolepis*, Lindl.8. *Eriobotrya*, Lindl.9. (*Photinia*, Lindl.).Section i. *Euphotinia*, Focke.ii. *Heteromeles*, Röm.10. *Pourthiaea*, Decsne.11. *Amelanchier*, Medic.12. *Stranvaesea*, Lindl.13. *Mespilus*, L., including *Crataegus*.14. (*Chamaemeles*, Lindl.)

The genera included in brackets have not been examined.

Chromosome Numbers in the *Pomoideæ*.

	Pollen mother-cell divisions.	Somatic chromosome number.
<i>Cotoneaster</i> " <i>procumbens</i> "	—	34
„ <i>congesta</i> , Baker	51/2	51
„ <i>rotundifolia</i> , Wall. (fig. 1)	—	68
„ <i>microphylla</i> , Wall.	—	68
„ <i>vulgaris</i> , Lindl.	34	—
„ <i>bullata</i> , Bois.	34	—
<i>Pyracantha</i> <i>Gibbsii</i> , A. B. Jackson	—	34
„ <i>coccinea</i> <i>Lalandei</i> , Dipp.	17	—
<i>Osteomeles</i> <i>Schwerinae</i> , Schneid	17	34
<i>Cydonia</i> <i>vulgaris</i> , Pers.	17	34
<i>Cydonia</i> (<i>Chaenomeles</i>) <i>japonica</i> , Pers.	17	34
„ <i>cathayensis</i> , Hemsl. (fig. 2) ..	17	—
„ <i>Maulei</i> , T. Moore	17	—

Chromosome Numbers in the Pomoidae—continued.

	Pollen mother-cell divisions.	Somatic chromosome number.
<i>Pyrus communis</i> , L. vars. "Fertility"	—	34
" " Williams' Bon Chrétien"	17	—
" " Beurré Giffard"	17	—
<i>Pyrus Malus</i> , L. vars. "Rev. W. Wilks"	17	—
" " Baumann's Reinette" ..	17	—
" " Washington"	51/2	—
<i>Pyrus torminalis</i> , Ehrh.	17	—
" <i>Sorbus</i> , Gaertn.	17	—
" (<i>Sorbus</i>) <i>minima</i> , Hedl.	51/2	—
" <i>Aucuparia</i> , L. (fig. 3)	—	34
" (<i>Sorbus</i>) <i>Aria</i> , Ehrh.	17	—
" (<i>Aronia</i>) <i>arbutifolia</i> , L. (fig. 4)	34	68
" <i>hybrida</i> , Moench.	17	—
<i>Rhapiolepis Delacourii</i> , André (fig. 5)	17	34
" <i>indica</i> , Lindl.	17	—
<i>Eriobotrya japonica</i> , Lindl. (fig. 6)	—	34
<i>Photinia (Pourthiaca) villosa</i> , DC. (fig. 7)	34	68
<i>Amelanchier stolonifera</i> , Wiegand	—	68
" <i>laevis</i> , Wiegand	34	68
" <i>rotundifolia</i> , Roem.	—	68
" <i>canadensis</i> , Torr. and Gray	34	—
<i>Strawcesia Davidiana undulata</i> , Rehd. and Wilson	17	34
<i>Mespilus germanica</i> , L.	17	—
<i>Crataegus monogyna</i> , Jacq.	17	34
" <i>oxyacantha</i> , L.	17	34
" <i>stipulosa</i> , Stend. (fig. 8)	—	34
" <i>rotundifolia</i> , Moench.	—	68
" <i>monogyna</i> , var. <i>cabulica</i> , Hort.	51/2	—
" <i>chlorsarca</i> , Maxim.	17	—
" <i>cognata</i> , Sarg.	34	—
" <i>pruinosa</i> , Koch.	34	—
" <i>apposita</i> , Sarg.	34	—
" <i>pedicellata</i> , Sarg.	34	—
" <i>cordata</i> seedling	—	72

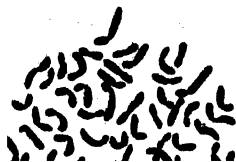


FIG. 1.—*Ostoneaster rotundifolia*
($2n = 68$).



FIG. 2.—*Cydonia cathayensis*
($2n = 34$).



FIG. 3.—*Pyrus Aucuparia*
($2n = 34$).

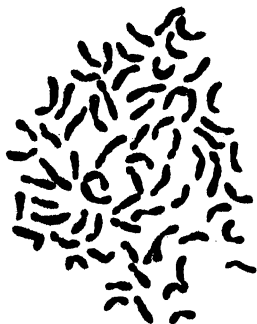


FIG. 4.—*Pyrus arbutifolia*
($2n = 68$).

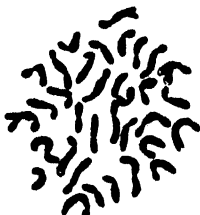


FIG. 5.—*Rhapsiolepis Delacourii*
($2n = 34$).



FIG. 6.—*Eriobotrya japonica*
($2n = 34$).



FIG. 7.—*Photinia villosa*
($2n = 68$).



FIG. 8.—*Crataegus stipulosa*
($2n = 34$).

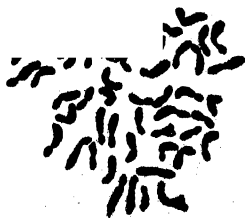


FIG. 9.—*Crataegus cordata*
seedling ($2n = 72$).

FIGS. 1-9.—Somatic divisions from root tip. ($\times 4000$.)

The observations show that the basic number of 17 runs throughout all the of the *Pomoideæ* examined and that all the species and varieties are orthoploid, having 34, 51 or 68 chromosomes. As will be shown later in this paper, aneuploid forms do occur in the progeny of triploids and tetraploids

(e.g., the seedling of *Crataegus cordata*), but these are usually of a weak constitution and probably incapable of supporting themselves under natural conditions.

Throughout the whole group the morphology of the chromosomes shows a great similarity. The chromosomes are all small, the largest being about 3 μ long and the smallest less than 1 μ . Types occur with median and submedian constrictions. It will be noted with reference to the illustrations that one type—the longest, a subterminally constricted chromosome—is represented four times in the diploid, six times in the triploid and eight times in the tetraploid. These chromosomes can, of course, only be identified with certainty where the fixation has been satisfactory.

Pollen Mother-cell Divisions.

(a) *General Remarks.*—Material of the following forms was examined:—

Diploids—

Pyracantha coccinea Lalandei, Dipp. *Cydonia* (*Chaenomeles*) *cathayensis*, Hemsl.; *C. japonica*, Pers.; *C. Maulei*, T. Moore. *Pyrus communis*, L. vars. "Fertility," "Williams' Bon Chrétien," and "Beurré Giffard." *Pyrus Malus*, L. vars. "Rev. W. Wilks," and "Baumann's Reinette." *Pyrus torminalis*, Ehrh.; *P. Sorbus*, Gaertn.; *P. Aria*, Ehrh.; *P. hybrida*, Moench. *Rhapiolepis Delacourii*, André; *R. indica*, Lindl. *Stranvæsia Davidiana undulata*, Rehd. and Wilson. *Mespilus germanica*, L. *Crataegus oxyacantha*, L.; *C. chlorosarca*, Maxim; *C. monogyna*, Jacq.

Triploids—

Cotoneaster congesta, Baker. *Pyrus Malus*, L. var. Washington; *P. minima*. *Crataegus monogyna*, var. *Cabulica*, Hort.

Tetraploids—

Cotoneaster vulgaris, Lindl.; *C. bullata*, Bois. *Pyrus arbutifolia*, L. *Photinia villosa*, DC. *Amelanchier canadensis*, Torr. and Gray; *A. laevis*, Wiegand. *Crataegus cognata*, Sarg.; *C. pruinosa*, Koch; *C. appposita*, Sarg.; *C. pedicellata*, Sarg.

The majority of the observations were made on the metaphase of the first pollen mother-cell division. Polar views were found to be particularly good in giving a clear general impression of the frequencies of different kinds of pairing. Physical connections between the chromosomes—where they occur—

cannot be seen in such plates, as they lie for the most part axially in the spindle and are therefore invisible in polar views. In side views of the metaphase it may be determined whether the chromosomes are associated or whether their proximity to one another is due to secondary pairing.

In the tetraploid species most of the observations are on polar views. In side views, on account of the large number of chromosomes, it was almost impossible to determine the nature of the individual associations.

In many instances chromosomes were found lying together, apparently associated, but it was impossible to determine whether these were real associations or were due to accidental placing of the chromosomes. In the triploids some of the associations found were of such a complicated nature that it was impossible to interpret them.

Pollen Mother-cell Divisions in Diploid Forms.

Throughout the genera of the Pomoideæ the diploid species all show a high degree of similarity in their chromosome behaviour at meiosis. At the metaphase of the first division the separation of the chromosomes is usually perfectly regular, 17 bodies being found passing to each pole. Abnormalities, such as the lagging of a univalent after the other chromosomes have passed to the poles, were found in a small percentage of cases.

In polar views of the first metaphase it was observed, in every division examined, that a certain number of bivalents were paired, giving groups of four and six chromosomes (figs. 10-21, and Plate 17, figs. 1-3). The extent of the pairing varies from three quadrivalent groups to the formation of three sexivalent groups and four quadrivalent groups (fig. 22). This was the maximum association observed, and it will be noticed that the 17 pairs of chromosomes appear as seven groups. It is impossible to determine whether the groups of chromosomes observed in polar view are true associations or are due to "secondary pairing." In side view of the metaphase, less abundant but more definite evidence of the nature of these groups of chromosomes may be found. Quadrivalent associations do occur, although not with such frequency as would appear to be indicated from the evidence of the polar views; but frequently in side views similar types of chromosomes may be observed lying in close proximity, without having any physical connections (figs. 24-27). Lawrence (1931) has made similar observations on *Dahlia*, and it is considered that the majority of the groups of chromosomes observed in polar views are due to this "secondary pairing," that is, they do not arise from the pairing of the chromatin threads at prophase, but from an attraction between the

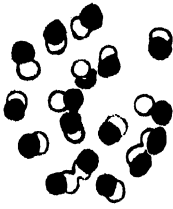


FIG. 10.—*Pyracantha coccinea*
Lalandei.



FIG. 11.—*Osteomeles*
Schwerinae.

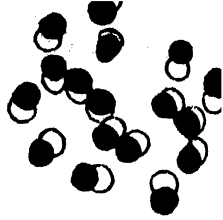


FIG. 12.—*Cydonia*
cathayensis.



FIG. 13.—*Pyrus communis*
var. "Fertility."



FIG. 14.—*Pyrus*
Sorbus.



FIG. 15.—*Pyrus*
Sorbus.



FIG. 16.—*Pyrus Aria*.

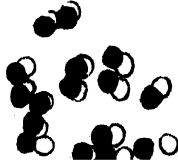


FIG. 17.—*Pyrus hybrida*.

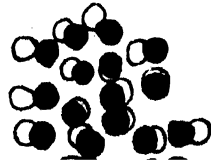


FIG. 18.—*Rhapsiolepis indica*.

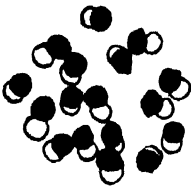


FIG. 19.—*Stranvaesia*
Davidiana undulata.

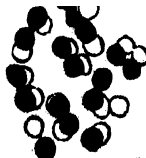


FIG. 20.—*Crataegus*
oxyacantha.

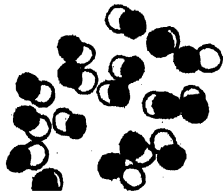


FIG. 21.—*Crataegus*
chlorosarca.

FIGS. 10-21.—Polar views of the metaphase of the first division, showing secondary pairing of chromosomes. ($\times 5000$.)

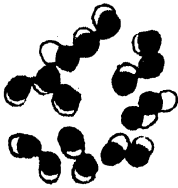


FIG. 22.—Apple "Northern Spy"; polar view of the first metaphase, showing the maximum pairing observed. Three groups of six chromosomes, two groups of four chromosomes. ($\times 5000$.)

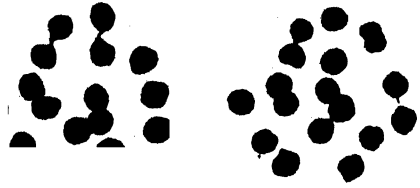


FIG. 23.—*Pyrus Aria*.—Early anaphase of the first division; the two plates drawn side by side showing similar groups of chromosomes in each. ($\times 5000$.)



FIG. 24.—*Pyrus Aria*.—Two quadrivalents. Similar types of chromosome lying together.



FIG. 25.—The same. Early anaphase.



FIG. 26.—*Cydonia cathayensis*, early anaphase. Quadrivalent dividing late.



FIG. 27.—*Crataegus monogyna*.—Similar chromosomes lying in close proximity.

FIGS. 24-27.—Side views of metaphase or early anaphase of the first division in diploids. ($\times 4000$.)

chromosomes at a later stage. Further evidence that the chromosome grouping observed at metaphase is of a "secondary" nature is obtained from observations of diakinesis. In the great majority of divisions examined 17 bivalents were observed, though in a small percentage multivalent associations were found (figs. 28-29).

In the two sides of the division at early first anaphase similar groupings of chromosomes are frequently observed (fig. 23). Fixation at this stage is usually very good, and the occurrence of these similar groups of chromosomes in comparatively widely separated plates, together with the fact that the groups at this stage and at metaphase are never less than seven and never include more than six chromosomes in a group, show that the phenomenon of secondary pairing is determined by the constant qualities of the chromosomes and is not merely an accident of fixation.

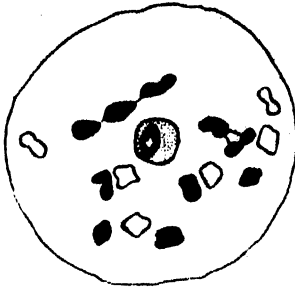


FIG. 28.—*Cydonia Maulei*.—One sexivalent, one quadrivalent, twelve bivalents. ($\times 5000$.)



FIG. 29.—*Cydonia cathayensis*.—One quadrivalent and fifteen bivalents. ($\times 5000$.)

FIGS. 28 and 29.—Diakinesis.

Prophase Stages.

At early diakinesis, bivalent chromosomes are found to be associated by chiasmata which are for the most part interstitial (fig. 30, A-N). This observation agrees with my interpretation of metaphase, where the chromosomes are frequently seen to be associated interstitially in a way described in *Prunus* by Darlington (1930). The same kind of relationship is seen in multivalent association in polyploids (fig. 30, O-Q).

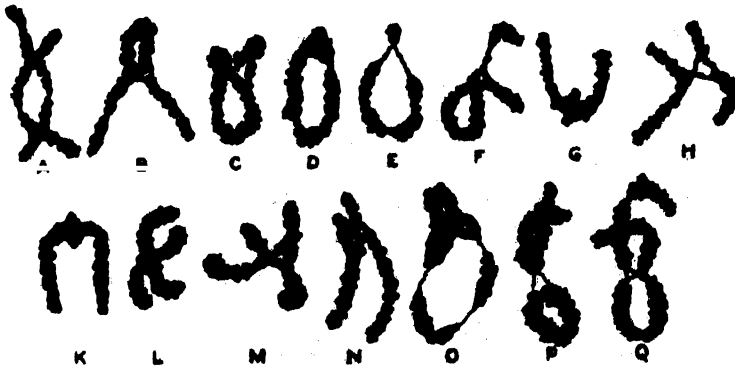


FIG. 30.—Prophase stages: A-N from diploids, O-Q from triploids. A-C, E-H, *Cydonia cathayensis*. D, K-N, *Rhapiolepis indica*. O, P, Q, *Pyrus minima*: O and P quadrivalents, Q pentavalent. ($\times 4800$)

5 (b). Pollen Mother-cell Divisions in Triploid Forms.

Two types of triploids may be distinguished according to their behaviour at meiosis:—

- (1) Forms in which the pairing is almost complete, rarely more than three or four univalents being found lagging at anaphase (as in *Pyrus Malus*, 1930).
- (2) Forms in which pairing is not so regular, as many as 14 univalents frequently being observed at first anaphase.

Crataegus monogyna var. *cabulica*, *Cotoneaster congesta* and *Pyrus Malus* var. "Washington" were found to belong to the former class. The commonest multivalent association is the trivalent, but higher combinations have been identified both in side and polar views of the first metaphase, indicating that autosyndesis takes place within each of the three sets of the triploid (figs. 31-33, 37). At first anaphase usually two or three univalents are found lagging, though as many as nine have been observed. Some of the multivalents observed were of such a complex nature that it was impossible to interpret them. These triploid forms evidently arise, as Rybin (1926) has shown in apples, through the functioning of an unreduced gamete in a normal diploid and not from a cross between a diploid and a tetraploid. These forms are therefore, like the triploid apples, non-hybrid or auto-polyploids.

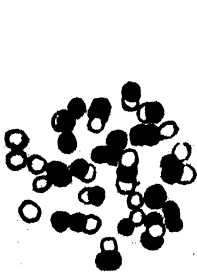
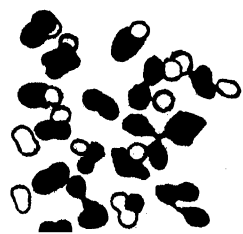
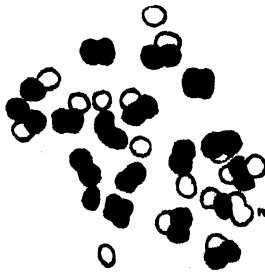


FIG. 31.—*Cotoneaster congesta*.



FIGS. 32 and 33.—*Crataegus monogyna* var. *cabulica*.

FIGS. 31-33.—Polar views of first metaphase in triploids. ($\times 5000$.)

Pyrus minima belongs to the second class of triploids.

Pyrus minima, a native British species found growing on Brecon Beacon, proved to be a triploid. It is doubtful if the plant examined was the true species, since being a triploid it would be incapable of maintaining itself by seed in the wild condition. It seems more probable that the material examined was from a seedling arising from a cross between a diploid and a

At meiosis as many as 16 univalents were observed at first metaphase, but counts at first anaphase of lagging univalents showed a mean of 13 (fig. 39).

The paired chromosomes at the first metaphase are grouped towards the centre of the plate (figs. 34-36), while the univalents lie in a ring round the

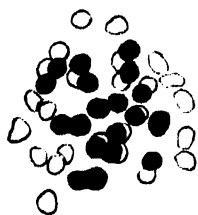


FIG. 34, 14 univalents.

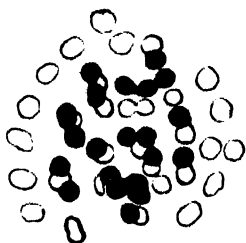


FIG. 35, 16 univalents.



FIG. 36, 13 univalents.

FIGS. 34-36.—*Pyrus minima*. Polar views of first metaphase in triploids. ($\times 5000$).

periphery. A similar type of arrangement of the univalents round the outside of the plate has been observed by Lawrence (1931) in *Dahlia*. Except for the occurrence of the univalents, the pairing of the chromosomes shows considerable similarity to that found in the "diploid" types of the Pomoideae. The paired chromosomes are associated into groups of four and sixes in a manner comparable with that described in the diploid (fig. 35). Trivalents do occur, but not with the frequency observed in autotriploid forms. Multivalent associations were found at early diakinesis (fig. 30, O-Q) showing that some of the higher associations are true multivalents due to the pairing of the chromosomes at prophase as opposed to secondary pairing. It is probable, however, that many of the groups of chromosomes observed in polar views are of a "secondary" nature.

At anaphase of the second divisions numerous univalents are found lagging (fig. 39).

Multivalent associations were found also in triploid apples (Darlington and Moffett, 1930) and they probably occur throughout the triploid forms, a much higher proportion of true multivalent associations occurring than in either diploid or tetraploid forms.

Such a condition may be explained by the behaviour of the triploid chromosomes at prophase. It has been shown in the triploid tulip (Newton and Darlington, 1929) that pairing can only take place between two of the three threads at any given point. This condition results in there always being a thread or part of a thread left free.

Since the "triploid" of the Pomoideae is really nonasomic for three chromosomes and hexasomic for four, taking a nonasomic type A, the chromosome

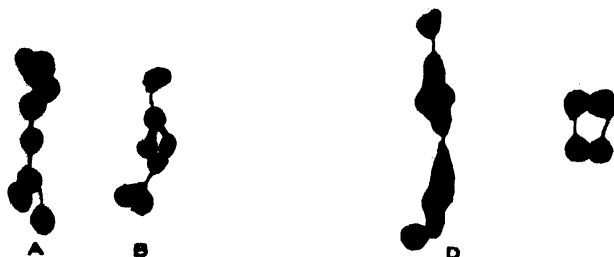


FIG. 37.—Multivalents from side views of the first metaphase in triploids. ($\times 6000$.) A-D, *Crataegus monogyna* var. *cabulica*. E, *Pyrus minima*. F, Apple variety "Washington." A, VIII. B, V. C, IV. D, VI. E, IV. F, V.



FIG. 38.—Anaphase of the first division in *Pyrus minima*. ($\times 4000$.) 12 univalents lagging to divide on the plate.



FIG. 39.—Anaphase of the second division in *Pyrus minima*. ($\times 4000$.) Chromosomes lagging on the plate.

occurs three times in each set of the triploid, and there is some difference in the degree of homology of each of these three.

The A chromosomes of the triploid may then be represented as follows :—

A_1	A_2	A_3
A_1	A_2	A_3
A_1	A_2	A_3

where 1, 2, and 3 represent the chromosomes of a particular "set" of the triploid. The chromosome A_1 will then pair with its homologues in a typical triploid manner as in the tulip and hyacinth, and the A_2 and the A_3 chromosomes will behave in the same way. In the pairing of the chromosomes to form a trivalent some portions of the chromosomes are not necessarily paired. As in the diploid *Primula floribunda* $\times$ *verticillata* (*Primula lewensis*) (Newton and Pellew, 1929) hybrid, a low degree of pairing may take place between the

unpaired portions of A_1 , A_2 and A_3 , thus giving rise to multivalent associations of a higher valency than trivalents.

If *Pyrus minima* arose from a cross between a diploid and a tetraploid, two of its chromosome sets (those from the tetraploid) would probably be similar, while the third set (from the diploid) would differ. The formula for such a triploid may be put down as follows, the figures indicating differences in homology and A representing one type chromosome of the three sets :—

$$\begin{array}{ccc} A_1 & A_2 & A_3 \\ A_1 & A_2 & A_3 \\ A_4 & A_5 & A_6 \end{array}$$

In such a plant A_1 would pair with A_1 , leaving A_4 free, and similarly A_2 would pair with A_2 and A_3 with A_3 , leaving A_5 and A_6 free. These free chromosomes may remain free as univalents, as appears usually to occur, or they may pair among themselves or with the other chromosomes as has been shown to occur in a low proportion of cases.

Pollen Mother-cell Divisions in the Tetraploids.

The divisions of meiosis show the abnormalities inherent in the multivalent association of chromosomes. One or two univalents are frequently found lagging at the anaphase of the first division, while as many as nine have been observed (*Amelanchier canadensis*).

The most frequent multivalent associations are quadrivalents, which are of the types found in *Prunus* (Darlington, 1928) and *Pyrus Malus* (Darlington and Moffett, 1930). Higher associations have been identified in the side views of the first metaphase (fig. 40), but the large number of chromosomes makes the

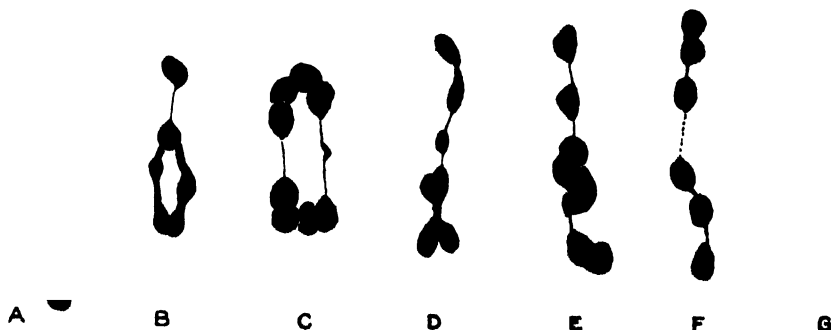
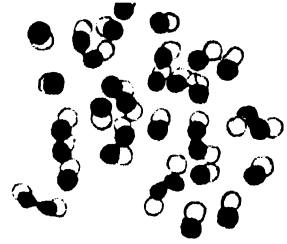
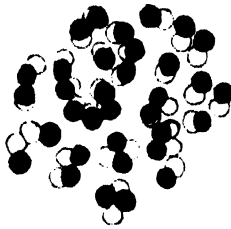
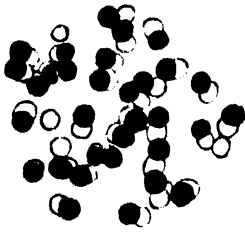
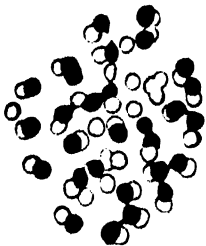
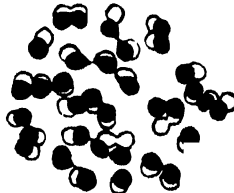
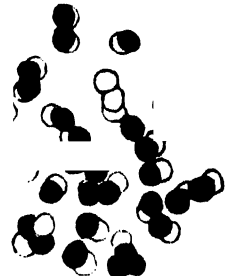
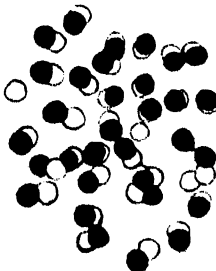
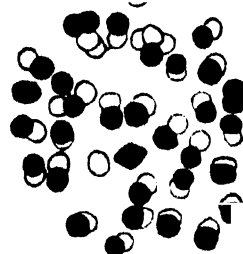


FIG. 40.—Multivalent associations from side views of the first metaphase in tetraploids. ($\times 6000$.) A-D, *Amelanchier canadensis*. E and F, *Cotoneaster vulgaris*. G, *Crataegus pedicellata*. A, B and D, VI. C, VIII. E, VIII. F, VI. G, V or possibly more.

interpretation of multivalents extremely difficult. Polar views of first metaphase give evidence of secondary pairing, groups of chromosomes being found which appear to be of a similar nature to those described in the diploids.

Apart from the quadrivalent associations, groups of six chromosomes are most frequently observed; but groups of eight and possibly 12 chromosomes are of common occurrence (figs. 41-48). From the nature of many of these

FIG. 41.—*Cotoneaster vulgaris*.FIG. 42.—*Cotoneaster vulgaris*.FIG. 43.—*Pyrus arbutifolia*.FIG. 44.—*Photinia villosa*.FIG. 45.—*Amelanchier canadensis*.FIG. 46.—*Crataegus appposita*.FIG. 47.—*Crataegus cognata*.FIG. 48.—*Crataegus cognata*.

FIGS. 41-48. Polar views of the first metaphase in tetraploids, showing multivalent association and secondary pairing. ($\times 5000$.)

groups it is obvious that it would be impossible to determine in side views whether they consist of secondarily paired chromosomes or are true multivalents.

At early anaphase of the first division it was observed that the chromosome groups in either plate frequently corresponded. If secondary pairing is an inherent property of the chromosomes themselves and is not merely a "clumping" due to fixation, this is to be expected. In *Pyrus arbutifolia* a particularly good example of similar groups in the two sides of anaphase was found (fig. 49). In this preparation the two sides of the anaphase were sufficiently widely separated to be in adjacent sections without any damage to either plate. Since correspondence of the chromosome groups in the two plates is almost complete, and it seems unlikely that fixation should clump the chromosomes

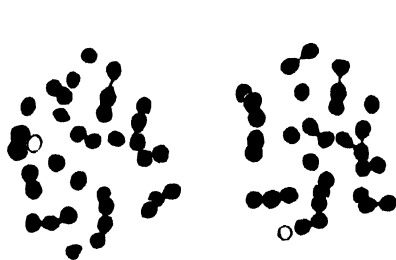


FIG. 49.—*Pyrus arbutifolia*.

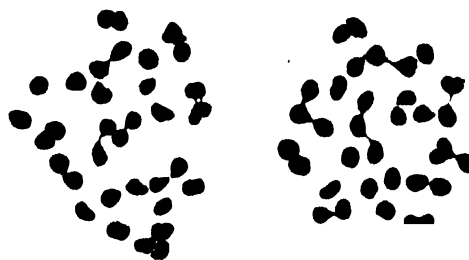


FIG. 50.—*Crataegus pruinosa*.

FIGS. 49 and 50.—Polar view of anaphase of the first division in tetraploids, showing similar groups of chromosomes in each plate. The two sides of the anaphase have been drawn side by side. ($\times 4000$.)

of two comparatively widely separated plates unless the chromosomes were already lying in definite groups.

Chromosome Numbers in the Progeny of Triploids.

Chromosome numbers were determined in 19 seedlings of crosses between diploid and triploid apples, and of 12 seedlings of triploid $\times$ triploid and triploids selfed. The seedlings were obtained from crosses made by Mr. M. B. Crane and Mr. W. J. C. Lawrence of this Institution.

The chromosome numbers are exceptional amongst the offspring of triploids. Previous observations on the progeny of selfing triploids or crossing them with diploids (as in *Datura*, Belling and Blakeslee, 1922; *Solanum*, Lesley, 1928) have indicated two distribution tendencies in respect of chromosome number:—

- (1) A binomial frequency of gametes with different numbers about the half number as mean.
- (2) The elimination of a proportion of male gametes and of zygotes with unbalanced numbers.

Table I.—Chromosome Numbers of Seedlings from Crosses between Diploids and Triploids.

	Number of chromosomes.										
	37	38	39	40	41	42	43	44	45	46	47
Lane's Prince Albert ($2n$) $\times$ Bramley's Seedling ($3n$)					1						
Bramley's Seedling ($3n$) $\times$ Cox's Orange ($2n$)				1			1				
Blenheim Orange ($3n$) $\times$ Cox's Orange ($2n$)				1	3		2				
Blenheim Orange ($3n$) $\times$ McIntosh Red ($2n$)	1				3	2		1	1		
Total	1		1	2	6	2	3	1	1		
Natural seedlings of Bramley's Seedling (probably $3n \times 2n$) from Darlington and Moffett's data, 1930.....		2	1	3	4		1				
Total number of seedlings	1	4	2	5	10	2	4	1	1	1	1

The seedlings here discussed are unusual among the progeny of triploids, and the additional numbers of seedlings support the previous evidence (Darlington and Moffett, 1930), that the highest frequency is observed with 41 chromosomes (fig. 51), which is the sum of the presumed primary haploid number

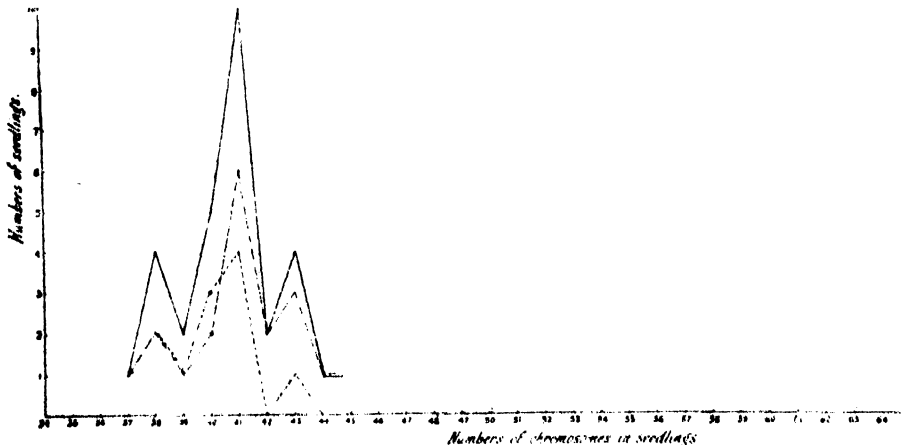


FIG. 51.—Diagram showing the frequency of chromosome numbers in seedlings from triploid apples.

Natural seedlings of Bramley's Seedling

Seedlings from crosses between diploids and triploids.....

Total number of seedlings from diploid and triploid crosses.....

Triploids $\times$ triploids or triploids selfed

(seven) and the secondary diploid number (thirty-four). It is to be imputed that the high frequency of seedlings with 41 chromosomes is due to the greater viability of the $17 + 7$ gametes in the triploid rather than to their zygotic viability, since seedlings having other chromosome numbers are frequently more vigorous than the 41 type.

The crosses between triploids show a greater frequency of plants with chromosome numbers higher than expectation on normal triploid segregation of chromosomes (fig. 51). This indicates a greater viability of zygotes with the higher chromosome numbers, but the number of seedlings examined so far is not sufficient to be significant.

Table II.

Number of chromosomes.

	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
Blenheim Orange × Ribston Pippin.....	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
Blenheim Orange selfed	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—
Ribston Pippin selfed	1	—	1	1	—	4	—	3	—	—	—	—	—	—	—	1
Total																

Secondary Pairing as Evidence of Chromosome Relation.

Since chromatin material is physiologically differentiated, chromosomes which pair together and segregate at random to produce homologous gametes must be themselves homologous. Cytological evidence indicates that different degrees of affinity exist between the homologous chromosome sets of polyploids, and under certain conditions, through the lack of competition, chromosomes that might be considered to be non-homologous are found to pair. Collins and Mann (1923) found complete chromosome pairing within the haploid set of *Crepis biennis*, by crossing it with *Crepis setosa*, and Darlington (1930) has shown a similar internal pairing in a cross between the hexaploid *Prunus domestica* and the diploid *Prunus cerasifera*. In *Primula floribunda* × *verticillata*, Newton and Pellew (1929) it was found in the diploid that the chromosomes from the two species paired regularly in absence of competition, whereas in the tetraploid the chromosomes of the two species always paired amongst themselves.

In the Pomoidæ it is probable that secondary pairing indicates a differential

affinity of the chromosomes similar to that indicated above, because the multivalent associations which occur in the triploid and occasionally in the tetraploid and diploid would be predicted on this basis.

In the diploid types we may distinguish two types of pairing :—

- (1) An almost universal formation of bivalents by prophase pairing.
- (2) A secondary superimposed pairing of these bivalents to form groups of four and six chromosomes each, seen only at metaphase and anaphase.

From the evidence of early diakinesis it has been shown that this grouping of the chromosomes at metaphase is for the most part purely of a "secondary" nature. Secondary pairing is evidently a criterion of relationship that can be applied to chromosomes that have differentiated by independent variation over a great period.

It is admittedly a difficult criterion to apply, but where, as in the present case, it is supported so fully by evidence of other kinds we are justified in drawing the conclusion that it indicated chromosome relationship.

The Pomoideæ as a Group in relation to Chromosome Constitution.

A. *Evidence*.—Cytological observations have shown that the *Pomoideæ* form a uniform group with the apparent basic number of 17. This conclusion was predicted on the basis of the hypothesis described for *Pyrus* (Darlington and Moffett, 1930). Longley (1924) working on *Crataegus*, and Meyer (1915) on *Mespilus*, found the basic number of these genera to be 16. I have examined 10 species and varieties of *Crataegus* in both somatic and pollen mother-cell divisions and in every case have found the basic number of 17. *Mespilus germanica* was examined in pollen mother-cell material and the basic number of 17 was found. It seems probable that the lower chromosome number assigned by these two workers to *Crataegus* and *Mespilus* is due to the occurrence of secondary association in a group of plants where polyploidy had not been suspected.

It is to the diploid forms that we must turn for the most critical evidence of autosyndesis within the group of 17, since in these forms the lower number of chromosomes and the absence of the further complication due to secondary polyploidy make a critical study more easy. As has been shown for *Pyrus* (*loc. cit. v. supra*, 1930), two types of association occur in the diploid forms—first, an almost invariable association of the chromosomes into 17 bivalents, and secondly, a variable superimposed association of these bivalents into three groups of three and four groups of two. In the triploids and tetraploids the

forms of association are more complex, but, throughout, associations of a higher valency are found corresponding with the sixes and fours of the diploid. These show that, as found in *Pyrus*, the original basic number of the Pomoideæ is less than the numerical basic number of 17, and the haploid or gametic chromosome number may be represented by the following formula as found for *Pyrus* :—

AAA
BBB
CCC
DD
EE
FF
GG

Such a condition has only been shown convincingly in one other species, *Dahlia Merckii* (Lawrence, 1929), where it is possible to point directly to the simple related tetraploid form within the genus. The chromosome constitution of the Pomoideæ cannot be interpreted on a primary basic number of eight, since if such were the case never more than one group of six secondarily paired chromosomes (three bivalents) would be found at metaphase, whereas two and three groups of six chromosomes are of common occurrence.

Further evidence is derived from the progeny of triploids. In the crosses between triploids and diploids it is found that the highest frequency occurs with 41 chromosomes (see p. 439). This result is in accordance with the usual rules of inheritance in aneuploids if we assume that the primary differentiation of the complement is into seven and not 17.

It is probable that secondary polyploidy is much more widespread than has hitherto been suspected, and that other secondary balanced forms will be found in the future.

Significance.—Throughout the Pomoideæ the chromosome complement is unbalanced relative to the balanced constitution found in other groups of the Rosaceæ. Since these new forms are capable of fulfilling the functions necessary for existence as well as the old types, they should be referred to as secondarily balanced, rather than unbalanced, types.

It has been shown experimentally that an unbalanced form always differs markedly from its progenitors in morphological characters, whatever the effect on its viability and fertility may be. Under experimental conditions unbalanced forms are usually found to have greatly reduced fertility and viability,

but that this is not always the case is clearly shown in *Dahlia Merckii* (Lawrence, 1929).

It would seem that out of the numerous unbalanced types capable of being produced in *Dahlia* and in the Rosaceæ a type can occur which is again balanced, but has a completely different chromosome constitution from its ancestors. Since in the Rosaceæ this change has produced a distinct and very widespread group (the Pomoideæ) it would appear that a change of chromosome constitution such as has been indicated constitutes a definite evolutionary step, and that such a group of plants will have a morphological expression different from that of the primary balanced form.

As a group the Pomoideæ are clearly distinguished from the rest of the Rosaceæ. Early workers divided the Rosaceæ into three groups—the Pomoideæ, Prunoideæ and Rosoideæ. This grouping of the Rosaceæ has since been supported by cytological evidence. The Pomoideæ have a basic number of seventeen, the Prunoideæ of eight and most of the families of the Rosoideæ have a basic number of seven. There are exceptions in the Rosoideæ, but these are of secondary importance.*

Within the group of the Pomoideæ, a high degree of similarity in certain morphological characters may be found throughout the genera. The most striking of these is the pome type of fruit, typical of all genera of the Pomoideæ, but not found outside the group. It seems plausible that this specialised type of fruit, peculiar to the group, may owe its evolution to the altered chromosome constitution found in the Pomoideæ.

Within the genera of the Pomoideæ an immense amount of variation is found, making a systematic study extremely difficult. Such a condition is to be expected from a group of complex polyploids, whose polyploidy furnishes a mechanism for the segregation of numerous variations, but whose general character is evidently determined by their characteristic balance.

Genetical studies of the Pomoideæ have been confined to the cultivated varieties of *Pyrus Malus*. Crane and Lawrence (1930) remark that in cultivated apples "incompatibility occurs in a varying degree, but is rarely, if ever, completely expressed in apples as in plums and cherries." Mr. Crane also tells me that the genetical work on apples is of a complexity quite distinct

* Kobel (1927) has reported the basic number of six in *Nuttallia cerasiformis*, Torr. et Gr. This species has been examined both in root tips and pollen mother-cells and the basic number of eight ($2n = 16$) has been found, bringing it into line with the rest of the Prunoideæ.

from that found in diploid fruits. Evidently this is to be attributed to the ancestral origin of the apples as polyploids.

[Note added May 19, 1931 :—

Secondary Pairing among Univalents.—Since going to press, further material of the triploid *Pyrus minima* has been examined. In this form about 13 univalents are usually found lying off the plate at the metaphase of the first reduction division. It was observed that the univalents frequently lie together in pairs scattered at random (fig. 52). This secondary pairing among univalents is to be expected if the 17 chromosomes are really composed of a primary set of seven. It would seem that the chromosomes within the set are sufficiently homologous to exhibit a mutual attraction but not sufficiently homologous to pair at prophase.]

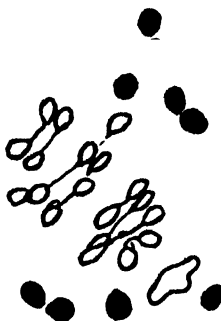


FIG. 52.—*Pyrus minima*. Side view of metaphase of the first reduction division, showing secondary pairing of univalents. The complete complement of chromosomes has not been drawn. ($\times 4000$.)

Summary.

(1) The basic chromosome number in the Pomoideæ is 17. All species and varieties examined were orthoploid (p. 426). Aneuploid seedlings from triploids are weakly.

(2) Diploid, triploid and tetraploid forms occur in the Pomoideæ. A particular long type of chromosome is represented four times in the diploid, six and eight times in the triploid and tetraploid respectively.

(3) Secondary pairing occurs amongst the chromosomes of all the "diploid" types of the Pomoideæ, forming sexivalent and quadrivalent groups. In extreme cases seven groups are formed—three sexivalents and four quadrivalents. Multivalent associations occur less frequently.

(4) Two types of triploids occur: (i) auto-triploids: these usually form trivalents, but higher associations have been observed, showing that auto-

syndesis takes place within each of the supposed haploid complements. Divisions are slightly irregular. (ii) Allo-triploids: formed from a cross between a diploid and a tetraploid, and having very irregular divisions with numerous univalents.

(5) Tetraploid forms show secondary pairing, groups of six and eight chromosomes being most frequently found.

(6) Seedlings of triploid $\times$ diploid apples most frequently had chromosome numbers of $2n + 7$, instead of giving a binomial frequency or elimination of intermediate numbers. In triploids intercrossed or selfed the seedlings showed a higher chromosome number than would be expected.

(7) Chromosome pairing, chromosome morphology and breeding results indicate that, as in *Pyrus*, the 34 chromosomes of the diploid Pomoideæ are of seven types, of which four are represented four times and three are represented six times.

(8) The number 17 is therefore a secondary basic number (unbalanced relative to the primary basic number) and the derived series of polyploids ($2n = 34, 51, 68$) are secondary polyploids.

(9) On analogy with experimental observations, the establishment of a secondary basic number must mean a definite evolutionary step. It is plausible, therefore, that the Pomoideæ owe their special morphological characters to this state of secondary balance. This indicates that change of balance is a factor in evolutionary change.

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DESCRIPTION OF PLATE 17.

FIGS. 1-6.—Microphotographs of the first pollen mother-cell division in diploid and tetraploid species of the Pomoideæ, showing secondary pairing.

FIG. 1.—*Cydonia cathayensis* ($2n = 34$). ($\times 2500$.)

FIG. 2.—Pear var. "Fertility" ($2n = 34$). ($\times 1700$.) Same as fig. 13.

FIG. 3.—*Rhapiolepis indica* ($2n = 34$). ($\times 2500$.)

FIGS. 4-6.—*Crataegus apposita* ($2n = 68$). Three photographs of the same plate at different levels. ($\times 1400$.) The same as fig. 46.

611.781:611.018

The Micelle Structure of the Wool Fibre.

By J. B. SPEAKMAN, Leeds University.

(Communicated by E. K. Rideal, F.R.S.—Received March 5, 1931.)

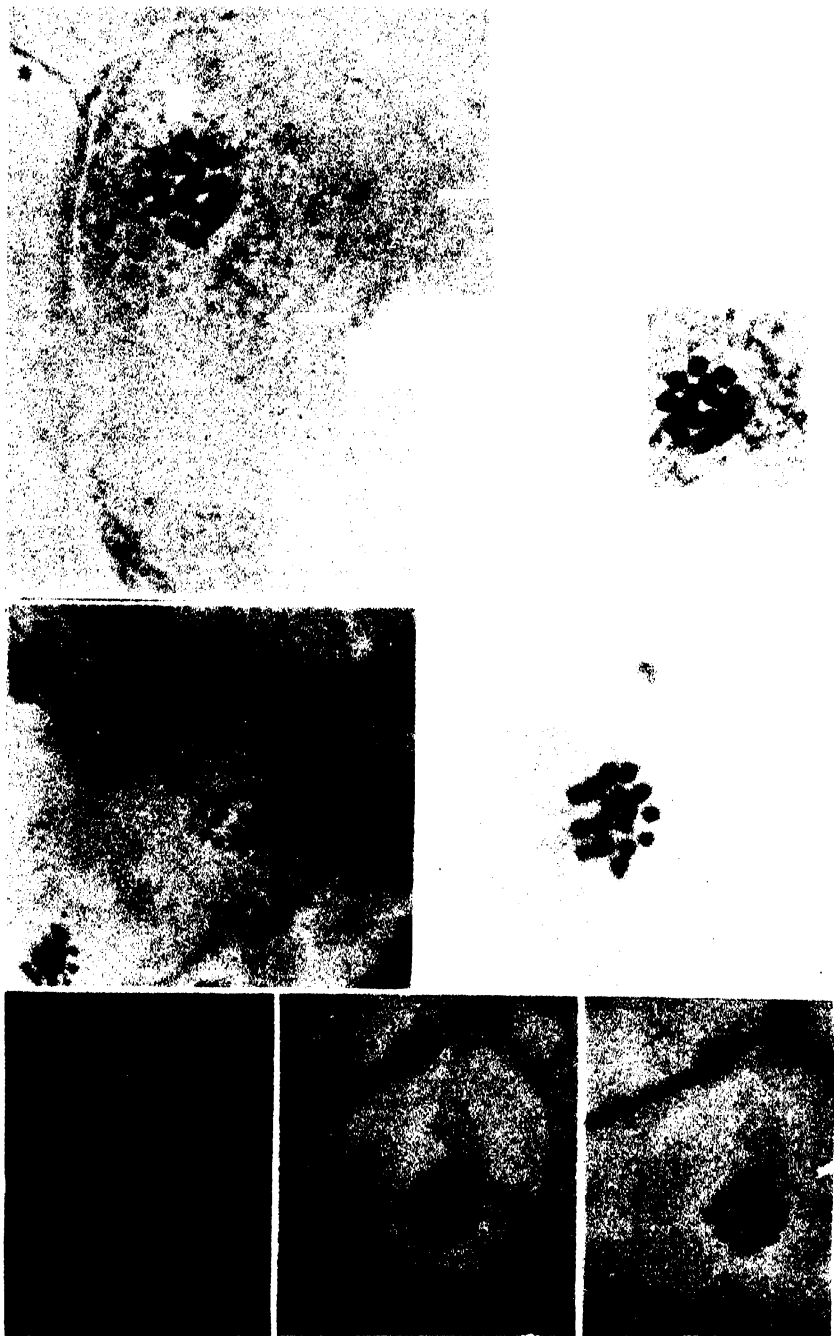
Abstract.

1. The wool fibre is constructed from micelles which are lamellar in shape. These are arranged with their long axes parallel to the length of the fibre and they are much longer, probably ten times longer, than they are thick.

2. The thickness of the micelles has been determined and is of the order of 200 Å.

3. The inter-micellar distance in the case of the dry fibre is small and of the order of 6 Å., but with wool fibres in water the distance is increased by swelling to 41 Å. Thus the swollen fibre is far more easily accessible to reagents of high molecular weight than the dry fibre.

(The full paper is printed in 'Proceedings' A, vol. 132, pp. 167-191.)



Investigations on Mediterranean Kala Azar. I.—Introduction and Epidemiology.

By S. ADLER and O. THEODOR.

(Kala Azar Commission of the Royal Society.)

(Communicated by H. H. Dale, Sec. R.S.—Received May 7, 1931.)

These investigations have been carried out with the support of the Anonymous Bequest Fund, administered by the Tropical Diseases Committee of the Royal Society. The services of Dr. Adler and Dr. Theodor, as well as important items of equipment and material, have been generously placed at the disposal of the Royal Society for this purpose by the Hebrew University of Jerusalem.

Visceral *Leishmaniasis* is distributed irregularly throughout the whole Mediterranean basin. Sporadic cases have been recorded throughout the whole area, but there are important foci where the disease is constantly present. The three largest foci are Catania (population 264,000 *circ.*, 150–200 cases annually), Naples (70 cases annually), and Palermo (70 cases annually), with their neighbouring villages. The disease is also constantly present in Tunis, Algeria, Spain, Malta, Greece (including Macedonia) and the Greek islands. In Palestine the disease is very rare, only one locally acquired case having been recorded during the last seven years. In districts where the disease is endemic its distribution is not uniform; there are always areas of maximum incidence, *e.g.*, near Naples the village of Bosco Trecase is more heavily infected than the surrounding villages. Endemic foci are often separated by large tracts of country where the disease is unknown; thus according to Pittaluga (1925) in Spain the eastern maritime provinces are infected, in the interior Madrid and Toledo are also infected, while the intervening country is free from the disease.

In the larger foci human and canine visceral *Leishmaniasis* always co-exist, but the relative incidence of the disease in man and dogs varies in different foci. In Catania, the largest of all endemic foci, Pulvirenti (1911) found an infection rate of about 1 per cent. in dogs; in Palermo, Jemma (1910) found no infections in 227 dogs, and Caronia and di Giorgio (1914) found none in 1,005 dogs examined. On the other hand, in Athens, which has relatively few cases of human visceral *Leishmaniasis* (about 20 cases a year in a population of nearly a million), Cardamatis (1912) found 13.8 per cent. of dogs infected.

It has been assumed that the canine and human diseases are identical, for the inoculation of the human parasite into dogs produces a disease indistinguishable from that occurring naturally in these animals, but the clinical signs of the natural infection in dogs vary in different centres. In Italy anæmia and emaciation are the only signs so far recorded. In Greece, Blanc and Caminopetros have discovered that the canine disease is frequently accompanied by cutaneous ulcers.* In four dogs, two of which we infected by the inoculation of bone marrow from human cases in Italy and two from the above two dogs, no ulceration was observed during an observation period of 7 to 10 months.

Jemma, di Cristina and Cannata (1910) in Palermo infected a dog with spleen juice of a human case and subsequently infected five other dogs from this animal. No cutaneous ulceration was recorded in any of the six animals during an observation period of 2 to 7 months.

The discrepancy between the distribution of human and canine visceral *Leishmaniasis* in different foci has yet to be investigated experimentally. Its cause in our opinion is to be sought in differences in the bionomics of the insect vectors in Italian and Greek foci of the disease.

Several important aspects of the epidemiology of the human disease in Italy have been investigated by the schools of Jemma in Palermo and Naples and of Longo in Catania. The disease is prevalent in the coastal region of Sicily and only a few sporadic cases have been found in the interior. It was established that the distribution of the disease is limited by altitude. Of 1,424 cases collected by Paradiso in Sicily none occurred above an altitude of 733 metres, and the large majority of cases occurred between sea level and 214 metres above sea level. The city of Enna and the village of Cesaro, 1,100 metres above sea level, have no endemic infantile Kala Azar although they lie within an endemic area. Pittaluga (1925) records a similar limitation of distribution in Spain, where no cases have been recorded above an altitude of 650 metres. In Catania Professor Longo and his colleagues have made a careful survey of the distribution of the disease, which was found to be confined almost entirely to the periphery of the town, while the thickly populated centre is hardly affected. This peculiarity in the distribution has also been observed in Naples, Palermo and in the Spanish foci of the disease. House to house infections are not the rule; although some streets are more heavily infected than others, there is usually an interval of 30 metres or more between one case

* Dr. L. Parrot, of the Pasteur Institute, Algiers, informs us that in Algeria 70 to 75 per cent. of all the dogs naturally infected with visceral *Leishmaniasis* develop cutaneous

and another. Family infections occur but are exceptional, and in some of the cases investigated it was possible to exclude infection by contact because of the long interval (3 years or more) between cases in the same family. The social status of the population appears to be of importance. The disease is commonest among the poorer rural or semi-rural population. Very few cases occur among the well-nourished children of the wealthier classes, who usually escape the disease even when residing in an endemic focus.

The most striking character of Mediterranean Kala Azar, differentiating it from the Indian form of the disease, is its age distribution. The following table is compiled from data collected by Paradiso (1926) in Catania for the years 1915-1925.

Table I.

	1-6 months.	6 months to 1 year.	1-2 years.	2-3 years.	3-4 years.	4-5 years.	5-10 years.	10-15 years.	Not stated.
es	1.47	11.8	48.74	17.42	8.15	3.23	3.23	0.49	5.48
	13.27								

Total number of cases, 1,424.

Total number of cases from Catania, 1,164.

Da Capua (1926) gives similar statistics for Naples and the surrounding countryside.

The following table is compiled from the data of Pittaluga for Spain for the years 1912-1924.

Table II.

	1-6 months.	6 months to 1 year.	1-2 years.	2-3 years.	3-4 years.	4-5 years.	5-6 years.	7-12 years.	12-46 years.	Not stated.
Per cent. of cases	3.2	10.5	34.9	16.9	14	10.2	4.7	1.2	1.2	3.2
	13.7									

Total number of cases, 343.

The above figures are in striking contrast to those collected by McCombie Young (1924) for Assam for the year 1922 on the basis of the number of treated patients.

Table III.

	Under 1 year.	1-5 years.	5-10 years.	10-15 years.	15-20 years.	20-30 years.	30-40 years.	40-50 years.	Over 50 years.
Per cent. of cases	0.06	8.59	20.5	20.2	16	16.9	9.6	2.9	0.37

Total number of cases, 17,724.

Although the incidence of the disease in children up to 5 years of age is larger in Assam, the number of cases under 1 year is comparatively negligible as compared to Catania.

In Sicily about 60 per cent. of all the cases occur in infants on the breast (among the poorer classes babies are breast-fed up to the age of two). The disease is rare in adults, from three to six cases annually being reported in Catania. Longo has suggested that Mediterranean adults are naturally immune to *Leishmania infantum*, and Maggiore (1923) has failed to infect adults by the inoculation of infected bone marrow, but he also obtained negative results after inoculating four babies with infected bone marrow and three with cultures 10 days old.*

There are no direct observations on the incubation period. So far no records have appeared of cases which developed the disease after a short stay in an endemic centre. Cases frequently come for treatment a month or more after the appearance of symptoms. There are many cases in which the incubation period is about 6 months or less, for the disease is not rare in infants of that age. On the other hand, incubation periods of less than 3 months are exceptional, for cases are very rare in infants of 3 to 6 months. Throughout the whole literature of the subject only one case has been recorded in an infant of 1 month (Paradiso, 1926).

The disease is prevalent throughout the whole year but in Catania is commonest in March and April.

In striking contrast to Mediterranean Kala Azar, the disease in India is characterised by—

- (1) the frequency of family infections ;
- (2) the frequency of house-to-house infections ;
- (3) the shorter incubation period (10 days to 3 months) ;
- (4) the rarity of infections in infants under 1 year ;

* Maggiore also obtained negative results after direct inoculation of *L. tropica* from sores into three babies. In Jerusalem we infected five out of six adults with *L. tropica* by direct inoculation from human lesions.

- (5) the heavier incidence of the disease during epidemics (up to 54 per cent. of the total population in an infected village (McCombie Young, 1924)).

In the Mediterranean foci the cases are constantly present and there is an almost uniform annual incidence without marked epidemic increases.

Points (1) and (2) clearly indicate differences in the bionomics of the vectors in India and the Mediterranean basin.

Point (3) may depend on a number of factors which require further investigation, e.g., racial differences in the human hosts, biological differences between *L. donovani* and *L. infantum*, or differences in the number of parasites inoculated during the act of transmission.

Clinically the diseases are practically identical. Their pathology is also very similar, but there are more parasites in the circulating blood of cases in India than in Italy.

This brief survey of the epidemiology of Mediterranean Kala Azar indicates that the transmission by the oral route can be practically excluded, for—

- (1) the disease is very rare in thickly populated urban slum districts where the conditions for transmission by contaminated food or water are ideal ;
- (2) it is common in breast-fed babies whose mothers are free from the disease.

Direct contact cannot play an important rôle in the spread of the disease, for family infections are rare.

The zoological relationship of *Leishmania infantum* to the genus *Leptomonas* of insects, as well as the epidemiology of the disease, are both in accord with transmission by a biting insect and cannot be fitted into any other mode of transmission. Although, as will be shown later, parasites can be demonstrated in the circulating blood of nearly all cases, they are sufficiently scarce to exclude mechanical transmission by insects through interrupted feeding. The relatively frequent occurrence of the disease in infants under 12 months indicates that transmission can occur in the absence of active interference on the part of the vertebrate host, i.e., directly through the bite of an insect. The insect vector of Mediterranean Kala Azar must fulfil the following conditions :—

- (1) It must have a bacteriologically sterile intestinal tract, as *Leishmania* cannot survive in the presence of bacteria. The adults of all species of sandflies hitherto investigated have a sterile alimentary tract. Accidental contamination with bacteria is fatal to sandflies.

- (2) Very small quantities of *L. infantum* should be able to produce an infection in the insect vector, because the number of parasites in the circulating blood is very small.

The first condition excludes mosquitoes as possible vectors, for although under artificial laboratory conditions it is possible to breed mosquitoes with a sterile alimentary tract, in nature these insects generally harbour a rich intestinal flora. Bed bugs have been infected with both *Leishmania donovani* and *Leishmania tropica*, but they have already been excluded on experimental grounds as natural vectors of these parasites, and their bionomics are incompatible with two important characteristics of Mediterranean Kala Azar, *i.e.*, the rarity of family infections and the absence of house to house infections, and moreover their distribution does not bear the slightest relationship to that of the disease.

The work of the Indian Kala Azar Commission (1925 *et seq.*) on the development of *L. donovani* in *Phlebotomus argentipes*, of the Royal Society Commission (1926 *et seq.*) with *P. chinensis*, and the observations of Adler and Theodor (1925 *et seq.*) on the development of *L. tropica* in *P. papatasi* and *P. sergenti* naturally drew attention to the possibility of transmission of Mediterranean Kala Azar by sandflies. Sinton (1925) suggested *P. perniciosus* as a possible vector because it was at that time the only known Mediterranean sandfly belonging to the *Major* group which also contains *P. argentipes*. Subsequent investigations have revealed the presence of several species of the *Major* group in the Mediterranean basin.

Adler and Theodor (1925) infected *P. papatasi* by feeding on cultures of *L. infantum* from various sources outside Italy. From July to September, 1929, the authors had an opportunity of studying sandflies in Naples and Catania. The general conclusion reached was that the distribution of the disease was compatible with transmission by *P. papatasi* and *P. perniciosus*. A quantitative study of the behaviour of Italian strains of *L. infantum* in *P. papatasi* showed that concentrations of 400 to 800 flagellates per centimetre produce practically no infection rate. With higher concentrations a number of infections was produced; the flagellates tended to an anterior position and persisted throughout the whole life of the sandfly. Qualitatively the behaviour of cultures of a *Leishmania* sp. is a true index of the behaviour of L.D. bodies of the same species of *Leishmania*. Quantitatively it is difficult to compare them, but in the case of *L. tropica* of Palestinian origin small numbers of flagellates produce high infection rates in *P. papatasi*. Since concentrations of 400 to 800 parasites per centimetre are never found in the circulating blood of infected

human beings, *P. papatasi* can be practically excluded as a carrier of the disease in Italy. *P. perniciosus* var. *tobbi* and *P. major* var. *syriacus* were infected by feeding on very small quantities of cultures of the Italian strains (1930). It therefore appeared that *P. perniciosus* would prove a more probable carrier than *P. papatasi*.

616.929.3

Investigations on Mediterranean Kala Azar. II.—*Leishmania infantum*.

By S. ADLER and O. THEODOR.

(Kala Azar Commission of the Royal Society.)

(Communicated by H. H. Dale, Sec. R.S.—Received May 7, 1931.)

[PLATE 18.]

The Leishman-Donovan bodies of *L. infantum* present no characters by which they can be distinguished from those of *L. donovani*, *L. tropica* or *L. brasiliensis*. In experimental animals they are either intracellular or lie in the fragments of dead cells. Individual L.-D. bodies may be distributed irregularly throughout the infected cells and their pseudopodia, each individual L.-D. body lying inside a definite vacuole, or they may be massed together in groups containing from 3 or 4 to 50 or 60 parasites. Each group of parasites is contained in a round or elliptical vacuole. In fixed or stained sections there is always a clear space between the wall of the vacuole and the periphery of the contained mass of parasites. These masses are formed by the ingestion of fragments of infected cells, for remnants of protoplasm of disintegrated cells can frequently be seen inside the vacuole.

Presence of L.-D. Bodies in the Circulating Blood.—In Indian Kala Azar L.-D. bodies have often been demonstrated in the circulating blood. Patton (1914) showed their presence in 100 per cent. of a total of 84 cases by the examination of blood smears. Cannata (1914) demonstrated the presence of L.-D. bodies in eight cases of infantile Kala Azar, but only after prolonged examination of a number of films, whereas Patton had demonstrated the presence of L.-D. bodies in 50 per cent. of his Indian cases by the examination

of a single film. Wenyon (1914) and Mayer and Werner (1914) among others demonstrated the presence of parasites in Indian Kala Azar by blood culture. The object of our researches was to determine roughly in how small a quantity of blood parasites could be detected. This is of obvious importance for any theory of insect transmission, as the infection of hæmatophagous insects from skin juices of human beings is hardly possible in Italy, where parasites have never been found in the skin. In a case of infantile Kala Azar acquired in Palestine it was found that cultures could be obtained by sowing 0.1 c.c. of blood on Locke-Blood-Agar.

In the following experiments varying amounts of blood were sown on Locke-Blood-Agar. The medium contained :—

Fresh rabbit blood	1 part.
Agar (3 per cent.)	1 part.
Locke solution (with 0.2 per cent. dextrose)	8 parts.

Three tubes were sown from each case (two with 0.1 and one with 0.03 c.c. of venous blood) and in 5 cases 3 additional tubes were sown with 2, 5 and 10 cmm. respectively.

While working with *L. tropica* it was found that medium made up with whole blood gave far better results than medium made up with serum for obtaining a primary culture. After isolation on Locke-Blood-Agar cultures can be carried on indefinitely on Locke-Serum-Agar, on which medium the rate of growth and cultural peculiarities can be readily observed.

Out of a total of 36 cases (34 examined in Italy during 1930 and 2 examined in Palestine during 1929) 33 gave positive blood cultures, i.e., 91.7 per cent. In 22 cases positive cultures were obtained from 0.03 c.c., in 3 cases from 5 cmm. and in one case from 2 cmm. of blood. Parasites could be demonstrated in 10 to 30 days by direct culture from the blood, while cultures of bone marrow gave positive results in 7 days.

Development of L. infantum in vitro.—The development of *L. infantum* was studied in hanging drops. Small pieces of spleen of infected animals (*Cricetus auratus* and *Microtus gümheri*) were placed in drops of Locke-Serum-Agar and kept at temperatures of 0°, 20°, 24° and 30° C. and observed daily.

The L.-D. bodies enlarge and multiply inside the dead macrophage. The eosinophile vacuole enlarges and the protoplasm of the non-motile protozoon becomes vacuolated and distinctly basophil. After 24 hours at 20° C. to 24° C. the flagellum protudes slightly beyond the body, and a few parasites show a to-and-fro movement inside the dead macrophage, but none show any marked

translatory movement. Occasionally large abnormal forms with three or four nuclei and blepharoplasts are observed. After 2 days there are many active flagellates with a smaller eosinophile vacuole (fig. 1). Many flagellates, including some of the larger forms, are still enclosed in dead macrophages. After 3 days the large flagellates up to $24\ \mu$ are numerous. This course of development is not uniform, for even after 2 days many individuals have not yet developed flagella. At 30°C . the development is more rapid. After 24 hours a number of parasites have flagella $2\text{--}3\ \mu$ long and a few are capable of translatory movement. After 48 hours many of the flagellates are $24\ \mu$ in length. Cultures made directly from tissues lived for 6 days at 30°C . Parallel observations were carried out with an Indian strain of *L. donovani* from the spleen of *Cricetus auratus*. At 20°C . active flagellates were not observed till the third day, but at higher temperatures the development was similar to that of *L. infantum*. The Indian strain died out on the third day at 30°C ., but this is not a specific character, as Christophers, Shortt and Barraud found one Indian strain which lived for 8 days at 30°C .

The Indian Kala Azar Commission (1926) classified the flagellates occurring in cultures into 6 types according to size. A strain of *L. donovani* from China isolated by the late Professor Young showed in culture forms up to $11.2\ \mu$, the majority being in the neighbourhood of $5\ \mu$. The organisms have either no flagella or flagella not more than 2 or $3\ \mu$ long. The culture therefore consists entirely of L.-D. bodies and type 2 of the Indian Commission, and no other types appear in cultures or in the stomach of *P. papatasi* infected by feeding on membranes. This strain when first isolated produced normal cultures, but during the time the strain has been in our laboratory it has produced only L.-D. bodies or flagellates with a stumpy flagellum.

In the case of *L. infantum* from Catania all the types described by the Indian Commission were found in the hanging drops, but the time of appearance of the various types was different. The Indian Commission found that all the types were present in a culture not earlier than the fifth day at a temperature of 22 to 24°C . The Catania strains showed all the types after 48 hours at a temperature of 24°C . and after 72 hours at 20°C . This may be due to essential

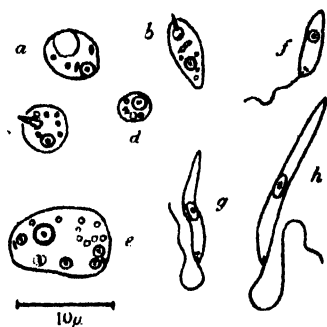


FIG. 1.—Developmental forms of *L. infantum* in culture. *a*–*e*, 28 hours old; *f*–*h*, 48 hours old.

differences between the Indian and Sicilian strains or to the fact that our observations were made with material from heavily infected spleens of *Cricetus auratus* and *Microtus g ntheri*, whereas those of the Indian Commission were made with human material from spleen and liver punctures.

Patton and Hindle (1927) found that Chinese strains of *L. donovani* resisted an intermittent temperature of -6 to 12° C. for 10 days. Tubes of Locke-Serum-Agar were inoculated with Catania strains of *L. infantum* and kept on ice for 10 days, after which they were put in the incubator at 24° C. and grew out normally. After being kept for 7 days on ice, fragments of infected spleen gave normal cultures of flagellates at temperatures of 24 to 30° C. There was no growth under anaerobic conditions at 24° C., and inoculated tubes kept anaerobically for 10 days showed only a few degenerated flagellates with a waving flagellum. After being kept for 10 days anaerobically tubes produced normal cultures when placed at 24° C. under normal conditions. It is a matter of experience that subcultures are always obtained if the inoculum contains a few flagellates with a waving flagellum even if the body of the flagellates are vacuolated and granular.

Serological Relations.—Using Noguchi's method of culture on medium made up with immune rabbit serum the Catania strains were found to be indistinguishable from Indian strains of *L. donovani* and Tunis strains of *L. infantum* and *L. canina*. All these strains produced many individual colonies on Locke-Serum-Agar made up with immune rabbit serum of Catania strains.

Leishmania infantum in Experimental Animals.—Experiments were carried out with geckoes, white mice, field mice (*Microtus g ntheri*) and hamsters (*Cricetus auratus* and *Cricetulus griseus*). An extensive literature already exists on the infectivity of *L. infantum* for animals. The objects of our experiments were the conservation of the virus in animals and particularly the feeding of sandflies on infected hamsters.

Geckoes.—Particular attention was paid to geckoes which have been suggested as possible reservoirs of human *Leishmaniasis*. Ed. and Et. Sargent, Lemaire and Senevet (1915) originally suggested them as reservoirs of *L. tropica*, and later Wenyon (1928) considered the possibility of their acting as reservoirs of *L. infantum*. Young and Hertig (1927) succeeded in infecting Chinese hamsters by inoculation of cultures of *L. tarentol *, the natural *Leishmania* of *T. mauritanica*, and it was therefore considered essential to inquire into the possible r le of geckoes as reservoirs of Mediterranean Kala Azar. It should be noted that Hindle (1930) failed to infect Chinese hamsters with *L. ceramodactyli* and *L. agam *, and concluded that there is no satisfactory experimental

evidence connecting geckoes with human *Leishmaniasis*. Catania is particularly suitable for this investigation, for the gecko *Tarentola mauritanica* is very common on the outskirts of the town where human cases are most numerous.

Fifty-one geckoes (49 *T. mauritanica* and 2 *Hemidactylus turcicus*) captured near Kala Azar houses were examined. In each case the heart was punctured through the skin with a fine capillary and a few drops of blood removed and sown on two tubes of Locke-Blood-Agar. Nearly all the animals survived the operation after which they were placed in jars covered with sandfly-proof gauze.

Cultures of *Trypanosoma ptyodactyli*, Catouillard (1909) were obtained from 17 out of 49 specimens of *T. mauritanica*, but all the geckoes were negative for *Leishmania*. It was thought that the growth of *Leishmania* might be influenced unfavourably by the presence of *T. ptyodactyli* and tubes of Locke-Blood-Agar were repeatedly sown with both *T. ptyodactyli* and one of the following strains of *Leishmania*: *L. tropica* (Palestinian strain), *L. infantum* (human strain from Catania), *L. tarentolæ* (Tunis strain which Young and Hertig found infective for hamsters) and *L. canina* (Tunis strain). In all cases the *Leishmanias* grew rapidly and produced rich cultures in 3 or 4 days, but very few trypanosomes were found. The above *Leishmanias* were also added to rich cultures of *T. ptyodactyli* with uniform results. The *Leishmanias* outgrew the *Trypanosome* and after 4 days only a few degenerating trypanosomes were found in a rich culture of *Leishmania*.

Attempts to infect geckoes by the inoculation of *L. infantum* from cultures and from infected bone marrow also gave negative results.

17.7.30. Two geckoes each inoculated intraperitoneally with 0.5 c.c. culture of *L. infantum* (11 days old).

29.7.30. Cultures taken from heart's blood. Both negative for *Leishmania*. One gave a culture of *T. ptyodactyli*.

29.7.30. Five geckoes inoculated each with 0.2 c.c. of bone marrow from a human case. The bone marrow showed an average of one L.-D. body per 20 fields. The geckoes had been in the laboratory for 10 weeks. Three of them were positive for *T. ptyodactyli*.

13.8.30. Cultures were taken from the heart's blood. All were negative for *Leishmania*. One gecko from which a culture of *T. ptyodactyli* was obtained 23.5.30, also proved negative for trypanosomes.

The absence of *Leishmania* from geckoes in a district where both geckoes and human infections are common proves that the gecko is not a factor in the aetiology of the disease as it occurs in Italy. It is to be noted that in other

localities geckoes have been found infected with *L. tarentolæ*. In Biskra the Sergents (1915) found 26·6 per cent. of geckoes, *T. mauretanica*, infected. In Tunis, Chatton and Blanc (1918) found 35·5 per cent. infected. It is significant that in Algeria and Tunis *P. minutus* is common. *P. minutus* has not been found in Sicily, and it is therefore possible that this species will prove to be the carrier of *L. tarentolæ*.

Mice.

Experiment No. 1—

- 18.5.30. Four white mice inoculated intraperitoneally each with 0·5 c.c. of culture (14 days old).
31.5.30. Inoculation repeated.
23.11.30. The animals died of a paratyphoid infection. Three were found to be infected with visceral *Leishmaniasis*.

Experiment No. 2—

- 27.5.30. Four mice inoculated subcutaneously into the tail with culture 15 days old.

Result.—No lesions on the tail during observation period of 10 months.

- 31.3.31. Spleen smears positive.

Chinese Hamsters, Cricetulus griseus.

Hamster No. 1—

- 13.5.30. Inoculated intraperitoneally with 0·5 c.c. of culture (12 days old).
21.5.30. Inoculated intraperitoneally with 2 c.c. of culture, 9 days old.
30.5.30. Inoculated intraperitoneally with 1·5 c.c. of culture, 30 days old.

Result.—18.6.30. The skin was infected. There were no cutaneous ulcers. The joints of the legs were swollen for 4 to 5 weeks and then returned to normal. The pathological changes are described below. Died 15.12.30 as a result of accident.

Hamster No. 2—

- 14.5.30. Inoculated intraperitoneally with 2 c.c. of infected bone marrow from four human cases.
17.5.30. Inoculated intraperitoneally with 0·5 c.c. infected human bone marrow.
21.5.30. Inoculated intraperitoneally with 0·5 c.c. of infected human bone marrow.

- 19.9.30. Sandflies *P. perniciosus* which fed on the animal were negative.
6.10.30. Inoculated intraperitoneally with 0.5 c.c. of infected human bone marrow.
9.10.30. Inoculated intraperitoneally with 1 c.c. of culture, 7 days old.
1.12.30. Inoculated intraperitoneally with 0.5 c.c. of culture, 15 days old.
18.12.30. Inoculated intraperitoneally with 0.5 c.c. of culture, 15 days old. Three weeks later blood culture was positive.

Syrian Hamster, Cricetus auratus, Waterhouse.

We are indebted to Mr. I. Aharoni, of the Department of Zoology of the Hebrew University, for this valuable animal, which he obtained and bred while on a zoological expedition in Northern Syria.

Mr. Aharoni gives the following details. One adult female and 12 young ones about 3 cm. in length were found on 12.4.30 $2\frac{1}{2}$ metres below the surface of the ground. The young animals were raised on a meat diet. Towards the end of July they were brought to Jerusalem and placed on a diet of vegetables and milk on which they thrived. 18.8.30, the first litter was produced. The size of the litter varies from 6 to 10. In nature this animal has a very limited distribution in the neighbourhood of Aleppo. Contrary to other species of the genera *Cricetus* and *Cricetulus*, the two sexes live together peacefully in captivity and they are therefore easy to breed.

- 20.10.30. Three hamsters inoculated intraperitoneally with 1 c.c. culture, 14 days old.
24.10.30. Inoculated intraperitoneally with 1 c.c. culture, 18 days old.
27.10.30. Inoculated intraperitoneally with 1 c.c. culture, 5 days old.
31.10.30. Inoculated intraperitoneally with 1 c.c. culture, 5 days old.
29.12.30. Partial splenectomy. Spleen smears showed a heavy infection in one animal and a slight infection in two animals.

Microtus g ntheri.

- 20.10.30 to 31.10.30. Two specimens received intraperitoneal injections as above.
29.12.30. Partial splenectomy. Spleen smears showed a heavy infection in both animals.

Pathology of Leishmania infantum.

The pathology of *L. donovani* infections in hamsters has been described in detail by Meleney (1925). Recently Hindle and Thomson (1928) have described the pathological changes caused by a Tunis strain of *L. infantum* in Chinese hamsters. The pathological changes produced by Catania strains of *L. infantum* in hamsters, *Cricetulus griseus* and *Cricetus auratus*, in field mice, *Microtus g ntheri*, and in white mice are radically different from those found by Hindle and Thomson. The latter observers found that after intraperitoneal and intratesticular inoculation the course of infection with a Tunis strain is as follows. A visceral infection is produced, parasites are present in the lymphatic glands, liver, spleen and blood stream. After a time the visceral infection diminishes or disappears and the infection becomes localised in the tissues surrounding the distal joints of the legs, tail and testes. The visceral infection almost disappears within 15 weeks after inoculation. The skin is not infected except in the neighbourhood of the swollen joints and superficial ulcers. So different are the changes produced by the Tunis strain from those found in infections with *L. donovani* from China, that the authors suggested that the Chinese and Mediterranean strains may be sufficiently distinct to be regarded as different varieties, if not species.

It may be remarked that periartritic lesions and ulcerations in the neighbourhood of mucous membranes are characteristic of *L. tropica* infections in mice following intraperitoneal injections, but in these animals there are also numerous parasites in the liver and spleen, and a visceral infection once established persists throughout the whole life of the animal.

A Tunis strain of *L. infantum* was found to produce a high infection rate in *P. papatasi* even with concentrations of flagellates one-tenth of those required to produce a low infection rate in the case of *L. infantum* of Italian origin. This Tunis strain resembles *L. tropica* in its behaviour more than other strains of *L. infantum* hitherto investigated.

In the case of Catania strains of *L. infantum* in Chinese hamsters, swellings of the joints of the hind legs were observed in one animal 5 weeks after inoculation, but these lasted only 4 weeks. Parasites were never found in smears of the peripheral blood; a few parasites, however, were present in the blood stream, for a culture was obtained from the blood of one hamster 7 months after inoculation.

In one animal the skin became infected within 5 weeks after inoculation and remained infected throughout its life. (The animal died 7 months after

inoculation.) During this period there was no ulceration. Infected macrophages were distributed throughout the whole skin and no part was found free from infection. In addition the skin was infiltrated with plasma cells and lymphocytes. There was no ulceration on the tail or mucous membranes. In *Cricetus auratus* the skin was found to be infected 2 months after the first inoculation, and no ulceration was observed during an observation period of 5 months. In *Microtus g ntheri* the infection of the skin is slight even when the visceral infection is intense (after 2 or 3 months), and large areas of skin are entirely free from infection. In white mice no skin infection was found even when the liver and spleen were heavily infected. In no case were L.-D. bodies found in blood smears.

Changes in the Viscera. Spleen.—In all the species the histological changes were similar and the following description is applicable to all. In the case of *Cricetus auratus* a partial splenectomy was performed on 29.12.30 (2 months after the first inoculation) on three animals, a portion of the spleen was examined and the remainder of the spleen was removed 2 months later. In one animal the spleen contained numerous parasites 2 months after inoculation, and in the remaining two the infection was slight; the spleen was equally enlarged in all three animals. Two months later the spleen was heavily infected in the two latter animals. Two specimens of *Microtus g ntheri* showed numerous parasites in the spleen 2 months after inoculation.

In *Cricetus auratus* the enlargement and tissue changes in the spleen are not necessarily dependent on the number of parasites present. The spleen may be markedly enlarged even when a few parasites are present. This point has some bearing on the pathological changes found in human beings who have relapsed after insufficient treatment. In these cases the spleen may be very large and the an mia severe; it may be necessary to do several spleen punctures before parasites are found. In such cases the prognosis is bad and the severity of the symptoms is proportional to the tissue changes, i.e., to the proliferation of macrophages rather than to the number of parasites in the body. In the earlier stages (after 2 months in *Cricetus auratus*) there is proliferation of reticulum cells, increase in the number of histiocytes and a large number of lymphocytes and plasma cells in the red pulp; the follicles are unchanged, but in later stages infected histiocytes also invade the follicles which may become almost obliterated. Infected cells are occasionally observed to phagocytise lymphocytes, polymorphs and erythrocytes. As the infection progresses the number of infected cells and plasma cells in the red pulp increases. Enormous numbers of plasma cells are typical of the later stages of *Leishmaniasis* in rodents.

Both the free histiocytes and the reticulum cells are infected with L.-D. bodies, but occasionally parasites may be found in neutrophil polymorphs. The distribution of parasites in the infected cells has already been described. Plasma cells, lymphocytes and infected macrophages from the red pulp enter the venous sinuses of the spleen. There is no evidence of destruction of L.-D. bodies either in the reticulum cells or in the histiocytes. The only cell in which destruction of L.-D. bodies may occur is the giant cell containing a number of nuclei near its periphery. These cells, which are formed by the fusion of infected cells, contain no L.-D. bodies in the centre and a varying number near the periphery in the neighbourhood of the nuclei. In heavily infected spleens of *Cricetus auratus* and *Microtus g ntheri* every gradation can be found between giant cells with many L.-D. bodies in the peripheral zone and with few or none. This process can have only a slight effect on the course of the disease, for giant cells are not numerous in the spleen at the height of the infection.

The extension of the infection in the spleen is due to the following factors :—

- (1) Proliferation of free histiocytes (round nucleus about 4 μ) and reticulum cells (elliptical nucleus 6 to 8 μ).
- (2) Infection through phagocytosis of fragments of infected cells and multiplication of the parasites.
- (3) Reticulum cells form direct communication with each other by means of protoplasmic processes, and L.-D. bodies may be carried from one cell to another along these communications. In cultures of spleen tissue Fischer and Dolschansky (1929) have actually observed direct interchange of cell contents by means of pseudopodia.

In advanced infections the majority of the infected cells are free histiocytes.

Liver.—The most characteristic change in the liver is the proliferation of free histiocytes (many of them infected), lymphocytes and plasma cells round the interlobular veins. As the infection advances the lymphocytes become less, and the plasma cells, more numerous. There is no infiltration round the intralobular veins (Plate 18, fig. 1). The Kupfer cells may contain numerous parasites, and there is extensive proliferation of endothelial cells both infected and non-infected in the capillaries (Plate 18, fig. 2). It is interesting to note that although in sections infected macrophages and plasma cells were found inside the interlobular veins, they were not found in the neighbouring intralobular veins. It is evident that the majority of the infected cells from the venous sinuses of the spleen enter the portal system but do not find their way into the general circulation. They either remain in the capillaries



FIG. 1.

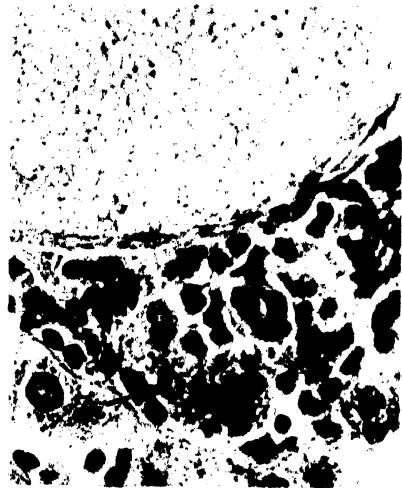


FIG. 2.



FIG. 4.



FIG. 5.

or wander out among the parenchyma cells of the liver. Only a part of the infected cells in the capillaries come from the venous sinuses of the spleen, as splenectomised white mice are susceptible to visceral *Leishmaniasis* (after intraperitoneal inoculation) and the liver is infected as usual. Very few parasites were found in the hepatic cells proper. Meleney found numerous parasites in these cells in Chinese hamsters in which the infection was of much longer duration than in our animals.

Lymphatic Glands.—The lymphatic tissue of the lymph glands may be almost entirely replaced by infected histiocytes and reticular cells.

The bone marrow contains numerous infected cells.

The adrenals are markedly enlarged (Plate 18, figs. 3, 4). In *Microtus g ntheri* they were three times as big as normal. The enlargement is confined entirely to the cortex and is due mainly to increase of the zona reticulata and hypertrophy of its cells. Finger-like processes of the zona reticulata grow down through the zona fasciculata towards the medulla and some cells of the zona reticulata are almost in contact with the medulla. No parasites were found in the adrenals.

The omentum contains a large number of infected histiocytes.

As the infection advances infected histiocytes may invade any tissue, e.g., in the Chinese hamster No. 1 many infected histiocytes were found among the cells of the heart muscle, kidney, small and large intestine, but in the other animals in which the observation period was shorter these organs were not affected.

From the above description it is evident that the course of infection of Catania strains of *L. infantum* in experimental animals is rather similar to that observed by Meleney in the case of Chinese strains of *L. donovani*.

In two specimens of *Cricetus auratus* infected with a strain of *L. donovani* from India the course of infection and the tissue changes were similar to those described above. Two specimens of *Microtus g ntheri* could not be infected with the Indian virus, even after repeated injections of large amounts of culture.

EXPLANATION OF PLATE 18.

FIG. 1.—Infiltration round interlobular vein (a) in liver of *Microtus g ntheri*. No infiltration round intralobular (central) vein (b). (Magn. 120 $\times$.)

FIG. 2.—Infiltration round interlobular vein in liver of *Microtus g ntheri* (a). Capillary stuffed with infected cells. (Magn. 620 $\times$.)

FIG. 3.—Adrenal of normal full-grown *Microtus g ntheri*. (Magn. 15 $\times$.)

FIG. 4.—Enlarged adrenal of *Microtus g ntheri* infected with *L. infantum*. (Magn. 15 $\times$.)

FIG. 5.—Group of flagellates of *L. infantum* (a) among teeth of epipharynx of *P. perniciosus* No. 578, 6 days after infecting feed. (Magn. 500 $\times$.)

Investigations on Mediterranean Kala Azar. III.—The Sandflies of the Mediterranean Basin. Distribution and Bionomics of Sandflies in Catania and District.

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(Kala Azar Commission of the Royal Society.)

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The diagnosis of preserved material is based on the characters of the pharynx, buccal cavity and spermathecae in the female, the pharynx, buccal cavity and external genitalia in the male. As has been shown (1930) these characters are not only essential for specific diagnosis, but they also form the basis of a natural classification. In fresh material the granules of the accessory glands of the female genitalia are sometimes an aid to diagnosis. The two closely related species *P. major* and *P. perniciosus* can readily be distinguished by these granules. In the former they are very small and coccoid, in the latter they are larger and more elliptical in form. The males of the erect-haired group can be determined without difficulty. The females of closely related species of the *Major* group are occasionally difficult to determine, and minor characters have to be employed. External characters are of little value for the diagnosis of living sandflies. In the case of the males, *P. papatasi* can be recognised by its relatively large external genitalia, and *P. sergenti* by its very short external genitalia. In the case of the females a few species, e.g., *P. perniciosus* and *P. major*, can be distinguished by their general appearance. Closely related species often differ widely in bionomics and it is essential to distinguish between them because in some cases they may differ in their capacity to transmit disease.

The sandflies of the Old World fall into two main groups, those which contain a well-marked armature in the buccal cavity and those which do not. These correspond roughly to the recumbent-haired and erect-haired groups of Newstead. The former group, i.e., the *Minutus* group, are not concerned in the transmission of human disease, for they feed almost exclusively on lizards. The only two species of this group which are known to feed on man are *P. schwetzi* and *P. babu*, which have not been recorded from Mediterranean countries. Nevertheless, the species of the *Minutus* group are included in the

following list, for they are probably concerned in the transmission of *L. tarentolæ* in geckoes.

P. papatasi, Scopoli, 1786.

Distribution : Ubiquitous throughout the whole Mediterranean basin.

P. sergenti, Parrot, 1917.

Distribution : Algeria, Tunis, Spain, Sicily, Syria.

P. perniciosus, Newstead, 1911.

A full re-description of this species and a discussion of its nomenclature and its relation to other species have been published recently (1931).

This sandfly has been recorded from Greece and Macedonia, but the identification is doubtful. We found no *P. perniciosus* in material from Greece in the possession of the London School of Tropical Medicine and the British Museum of Natural History. It is probable that this species has been confused with another closely related species, *P. macedonicus* n.sp. and a race of *P. chinensis*.

Distribution : Italy, Malta, France, Spain, Algeria, Tunis.

P. perniciosus, var. *tobbi*, Adler and Theodor, 1930.

Distribution : North Palestine, Persia.

P. langeroni, Nitzulescu, 1930, and *P. langeroni*, var. *longicuspis*.

Distribution : Tunis.

P. vesuvianus, Adler and Theodor, 1931.

Synonym : *P. laroussei*, Langeron and Nitzulescu, 1931.

This species was discovered in August, 1929, in Naples. In a re-examination of material of the Paris Faculty of Medicine which originates from Bourg la Reine and Varennes, Nitzulescu found some specimens and described them under the name of *P. laroussei*.

Distribution : Naples, France.

P. ariasi, Tonnoir, 1921.

This species was recently recorded from France (Saint Vallier, Alpes maritimes) and re-described by Nitzulescu.

Distribution : France, Spain.

P. major, Annandale, 1910.

Synonym : *P. neglectus*, Tonnoir, 1921.

Tonnoir described *P. neglectus* from Albania and Dalmatia. Sinton considered it a synonym of *P. perniciosus*, from which, however, it can be readily

distinguished. A comparison of specimens of *P. major* from India, Caucasus, Crimea and Italy with the types of *P. neglectus* showed that they are practically identical. There are a few slight differences in the palp formula, and the European form may be considered as a local race of *P. major*. Measurements of the European form have been given by Tonnoir and Nitzulescu.

Female.

Size : 2·2–3 mm.

Wings : Length, 2·5 mm. ; breadth, 0·8 mm. ; wing index, $\alpha/\beta = 1\cdot8$.

Palp formula : 1, 4, 2, 3, 5. Relative length of segments : 1,—4·4, —4·8,—3·5,—7. In the Indian form the palp formula is 1, 4, (3, 2), 5. In the four Indian specimens we examined the second segment is always a little longer than the third. Relative length of segments in the Indian form : 1,—5·4,—5·2,—3·5,—7.

Antennae : $3 > 4 + 5$.

Pharynx : (Fig. 1, b). In the posterior portion a system of teeth appearing in optical section as small points. The teeth are larger than those of *P. perniciosus* and extend very much further anteriorly. The pharynx is rather similar to that of *P. wenyoni*, which is closely related to *P. major*. Laterally the teeth appear as oblique scales which extend half way up the pharynx.

Spermathecae : Consisting of 10–12 segments with a long process. The ducts are rather wide at the base and narrow markedly in the distal third. Those of *P. perniciosus* and of known allied species are of uniform width.

Accessory glands : As previously mentioned, the granules of the glands are very small and coccoid ; in *P. perniciosus* they are much larger and elliptical.

The legs of *P. major* are very long and give the insect a very characteristic appearance by which it can be distinguished at sight from *P. perniciosus*. *P. major* is of light yellowish grey colour, very slender, standing on relatively long legs, while *P. perniciosus* is blackish, much smaller and has short stout legs.

Male.

Size, wings, palps and antennae as in the female.

Pharynx : Similar to that of the female. The teeth are much finer and do not extend as far anteriorly as in the female, but much more so than in *P. perniciosus*.

Genitalia : (Fig. 2) Similar to those of the Indian form. The two median spines of the distal segment of the superior clasper are situated near the junction of middle and apical third of the segment. In the Indian form they are slightly more distal, but this difference is not constant. In specimens from the Caucasus and the Crimea their position is the same as in the European form. The position of these spines and the palp formula are apparently characters which vary in different local races.

Distribution : Sicily, Dalmatia, Albania, Tyrol, Crete.

P. major, var. *syriacus*, var. nov.

Synonym : *P. major*, Adler and Theodor, 1929.

This species was previously considered to be identical with *P. major*. A comparison of material from Palestine and Syria with Indian and European specimens showed that there are constant differences which justify the creation of a variety.

Female.

Size : 2-2.5 mm.

Palp formula : 1, 4, 2, 3, 5. Relative length of segments : 1,—4.2,—4.6,—3.3,—6. The second segment is always markedly shorter than the third.

Antennæ : 3 + 4 + 5.

Pharynx : The teeth are smaller than and do not extend as far anteriorly as in the type form. The lateral teeth do not extend much further anteriorly than the median ones. The armature closely resembles that of *P. perniciosus*.

Spermathecae : The capsules are as in the type form but the ducts are uniformly narrow throughout their length.

Male.

Size, palps, antennæ as in the female. **Pharynx** similar to that of the female but the teeth are less developed.

Genitalia : Similar to those of the type form. The two median spines on the distal segment of the superior clasper are situated very near the proximal one.

The variety differs from the type form in its smaller size, the arrangement of the teeth in the pharynx and the position of the spines on the distal segment of the superior clasper.

Distribution : Syria, Palestine.

P. macedonicus sp.n.

This species has been confused with *P. perniciosus*, to which it is closely related.

Female.

Size : 2 mm.

Wings : Length, 1.9 mm. ; breadth, 0.6 mm. ; wing index, $\alpha/\beta = 1.7-1.8$.

Palp formula : 1, 4, 2, 3, 5. Relative length of segments : 1.—3.5,—4,—3,—5. The fourth and fifth segment are relatively shorter than the corresponding segments in *P. perniciosus*.

Antennæ : $3 > 4 + 5$.

Pharynx : (Fig. 1, a). Very similar to that of *P. perniciosus*. The teeth are arranged in transverse parallel lines which are not as convex as in *P. perniciosus*. They are larger than those of *P. perniciosus* and not as closely set.

Spermatheca : As in *P. perniciosus* with uniformly wide ducts.

Male.

Size, wings, palps and antennæ as in the female.

Genitalia : (Fig. 3). In general outline similar to those of *P. perniciosus*.

The two median spines on the distal segment of the superior clasper are situated rather near the proximal spine ; in *P. perniciosus* they are half-way between proximal and terminal spines.

Penis : The borders of the proximal two-thirds of the penis are almost parallel. The genital filaments emerge at the junction of the middle and distal third. Beyond this point the penis has the form of a shallow blunt hook, convex dorsally. Near the distal end there are three to five small sharp processes.

The female is very difficult to distinguish from *P. perniciosus*. The male differs from other species in the shape of the penis and in the position of the median spines on the distal segment of the superior clasper.

Distribution : Macedonia (Waterston), 4 ♀♀, 19 ♂♂.

P. canuuniticus sp.n.*Female.*

Size : 2.5 mm.

Wings : Length, 2.6 mm. ; breadth, 0.7 mm. ; wing index, $\alpha/\beta = 2$.

Palp formula : 1, 4, 3, 2, 5. Relative length of segments : 1,—4.9,—4.7,—4,—10.

Antennæ : $3 > 4 + 5$.

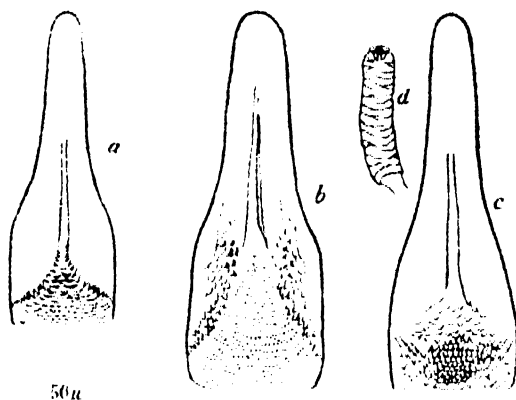


FIG. 1.—Pharynx of female of (a) *P. macedonicus*, (b) *P. major*, (c) *P. canaaniticus*, (d) Spermatheca of *P. canaaniticus*.

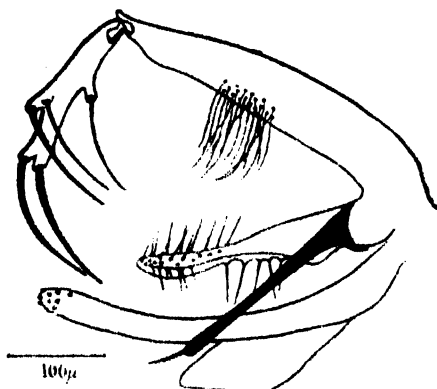


FIG. 2.—Male genitalia of *P. major*.

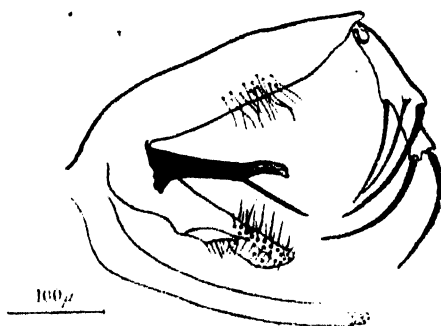


FIG. 3.—Male genitalia of *P. macedonicus*.

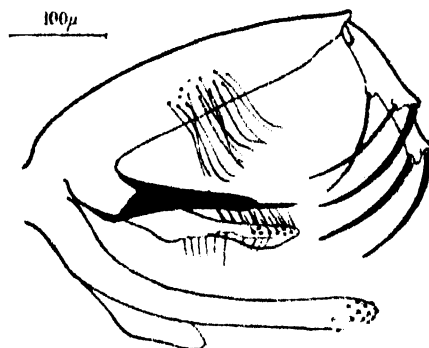


FIG. 4.—Male genitalia of *P. canaaniticus*

Pharynx : (Fig. 1, c). The armature consists of blunt broad teeth. The toothed area is roughly triangular. The teeth point towards the middle of the posterior border. They are very similar to those of *P. vesuvianus*, but are smaller and less prominent. In optical section the median teeth appear as scales while the lateral ones are wedge-shaped.

Spermathecae : (Fig. 1, d). The spermathecae are tubular and have a rudimentary segmentation similar to those of *P. chinensis* and *P. vesuvianus*. The capsules are of uniform width and not spindle shaped as in *P. chinensis*. They bear a very short process with small "hairs" projecting slightly beyond the tip of the capsule.

Male.

Size and antennae as in the female.

Wings narrower than in the female. Breadth, 0.5 mm.

Palp formula : 1, 4, 2, 3, 5. Relative length of the segments: 1,—3.7,—4.3,—3.3,—7. The palp formula differs from that of the female in that the third segment is longer than the second, while in the female it is slightly shorter.

Pharynx : Similar to that of female.

Genitalia : (Fig. 4). Similar to those of *P. major*. The two median spines on the distal segment of the superior clasper stand near the proximal one. The penis is much shorter than the median appendage. It narrows towards the extremity and has a round tip. The inferior clasper is slightly longer than the proximal segment of the superior clasper.

P. canaaniticus is closely related to *P. vesuvianus*, from which it differs in the size of the teeth in the pharynx and in the absence of a prominent projection on the spermathecae. It seemed doubtful whether the male and female described above could be included in the same species because of the different palp formulæ, but since the pharynx, a much more important character, is similar in both sexes and shows features which distinguish it from other members of the *Major* group, it was thought justifiable to consider them as one species.

Distribution : Palestine, Rosh Pinah, 2 ♀♀, 2 ♂♂. Tel-Aviv, 1 ♂. Ben Shemen, 1 ♂.

P. chinensis, Newstead.

Synonym : *P. major*, var. *chinensis*, Newstead, 1916. *P. major*, var. *longiductus*, Parrot, 1928.

No other sandfly tends to form local races to the same extent as *P. chinensis*. These races vary mainly in the shape of the penis and in the length of the genital filaments (fig. 5).



FIG. 5.—Penis of various races of *P. chinensis*. (a) Syrian and Persian race ; (b) Chinese race ; (c) Yugoslavian and Palestinian race ; (d) Roumanian race (after Nitzulescu).

Four races exist in the Mediterranean basin :—

- (1) A race in Syria in which the penis is broad and bears a pronounced angular subterminal tubercle. The genital filaments are very long.
- (2) A form recorded from Yugoslavia and Macedonia in which the penis is thin and conical and the subterminal tubercle is small but well marked. The filaments are relatively short.
- (3) A Roumanian form with long genital filaments and a flat tubercle more distant from the extremity than in the other forms. The penis resembles that of the Chinese form.
- (4) A dwarf form of the Yugoslavian race (2) confined to Galilee.

P. minutus, Rondani, 1843.

This species was originally recorded from Italy. Newstead recorded it from Malta. Sinton examined Newstead's specimens and found them to correspond to the re-description of *P. minutus* from Palestine (1925). *P. minutus* as described from Palestine was not found in Naples or Catania, and it is therefore probable that *P. parroti*, var. *italicus* is really the same as *P. minutus*, Rondani. This point cannot be determined as Rondani's types cannot be traced. Should *P. parroti*, var. *italicus* prove to be a synonym of *P. minutus*, considerable changes in the nomenclature of the *Minutus* group would be necessary. For the purpose of this paper the name *P. minutus* is used for the species described from Palestine.

Distribution : Palestine, Malta, Algeria.

P. parroti, Adler and Theodor, 1927.

Distribution : Algeria, Tunis, Spain.

P. parroti, var. *italicus*, Adler and Theodor, 1931.

Distribution : Naples and environments, Sicily.

P. africanus, Newstead, 1912.

Distribution : Palestine, East Africa, Congo.

P. fallax, Parrot, 1921.

Distribution : Algeria.

P. palestinensis, Adler and Theodor, 1927.

Distribution : Palestine and Persia.

Key to the Sandflies of the Mediterranean Basin.

Females.

1. Well-marked armature and pigmented area in buccal cavity..... 2
No armature in buccal cavity..... 6
2. Spermathecae tubular 3
Spermathecae capsules with narrow ducts..... 5
3. Teeth in buccal cavity pointed, median ones narrower than lateral ones standing on an arc convex anteriorly..... *P. minutus*
Teeth in buccal cavity all of equal size arranged in a straight line..... 4
4. Number of teeth, circ. 70-80..... *P. parroti*
Number of teeth, circ. 50..... *P. parroti italicus*
5. Circ. 20 teeth in buccal cavity standing on a straight line, capsules of spermathecae almost spherical *P. palestinensis*
Circ. 40-50 teeth in buccal cavity standing on an arc slightly convex anteriorly. Spermathecae elongated capsules *P. africanus*
6. Spermathecae completely segmented bearing a long process distally. Teeth in pharynx arranged in parallel rows of points..... 7
Spermathecae completely segmented bearing a knob distally..... 10
Spermathecae incompletely segmented 11
7. Toothed area in pharynx not extending beyond posterior third..... 8
Toothed area covering more than posterior third of pharynx, teeth laterally appearing as oblique scales extending further anteriorly than the median ones..... 9
8. Process at distal end of spermathecae, short and thick. Segmentation irregular
P. ariasi
Process long and slender. Segmentation complete. Ducts uniformly wide.
P. perniciosus, *P. perniciosus* var. *tobbi*, *P. macedonicus*
9. Ducts of spermathecae uniformly narrow. Teeth in pharynx fine and extending slightly beyond posterior third *P. major*, var. *syriacus*
Ducts of spermathecae wide at base, narrowing in terminal third. Teeth in pharynx coarser and occupy posterior half of pharynx..... *P. major*
10. Spermathecae consisting of 10-12 equal segments. Armature in pharynx appears as a network of lines..... *P. papatasi*
Spermathecae consisting of 4-5 segments, distal segment larger than the rest. Teeth in pharynx very large with axis vertical *P. sergenti*

11. Toothed area in pharynx distinctly triangular, teeth wedge shaped. Spermathecae spindle shaped..... *P. chinensis*
Teeth blunt. Spermathecae broad at the end 12
12. Teeth in pharynx large. Spermathecae bearing a prominent knob distally
P. vesuvianus
Teeth in pharynx smaller. Knob very small, projecting very slightly beyond distal end of spermathecae *P. canaaniticus*
The females of *P. fallax*, *P. langeroni* and *P. langeroni* var. *longicuspis* are unknown.

Males.

1. Four thick spines on distal segment of superior clasper..... 2
Five spines on distal segment of superior clasper 6
2. Armature in buccal cavity absent. Brush of hairs on proximal segment of superior clasper. Distal segment elliptical, 2 spines terminal, 2 at proximal third of segment *P. sergenti*
Armature in buccal cavity present. No brush on proximal segment of superior clasper. Distal segment cylindrical. Four spines terminal or 2 sub-terminal, a very thin anterior fifth spine present..... 3
3. Penis pointed, conical..... *P. africanus*
Penis, thick, finger-like 4
4. Teeth in buccal cavity standing on an arc convex anteriorly..... 5
Teeth very small and irregular, standing on a straight line..... *P. parroti*
5. Distal segment of superior clasper 7-8 times as long as broad, fifth small spine sub-terminal *P. fallax*
Distal segment of superior clasper 4-5 times as long as broad, small fifth spine near distal third of segment *P. minutus*
6. Distal segment very long, 3 short spines terminal, 2 at distal third of segment. Median appendage with 2 long processes *P. papatasi*
Distal segment shorter. 2 long spines terminal, 3 long ones at about middle of segment. Median appendage simple 7
7. Termination of penis bifid 8
Termination of penis not bifid 9
8. Both points of fork subequal. Two medium spines on distal segment of superior clasper half way between proximal and terminal spines..... *P. perniciosus*
One point of fork much broader and longer than the other, 2 median spines very near proximal one *P. perniciosus*, var. *tobbi*
9. Penis with subterminal tubercle. Genital filaments emerging from tip... *P. chinensis*
Genital filaments emerging at distal third of penis which ends in a long curved hook with several small processes near distal end..... *P. macedonicus*
Penis very long and slender with round tip without subterminal tubercle..... 10
End of penis truncated and pointed..... 12
10. Penis almost as long as median appendage. Teeth in pharynx appearing as rows of points 11
Penis tapering towards end, markedly shorter than median appendage. Teeth in pharynx appearing as broad scales..... *P. canaaniticus*
Penis short and broad with thick club-shaped extremity..... *P. ariasi*
11. Teeth extending almost half way up the pharynx..... *P. major*
Toothed area occupying about posterior third of pharynx..... *P. major syriacus*

12. Terminal pointed part of penis short..... *P. langeroni*
 Terminal pointed part of penis long..... *P. langeroni longicauspis*
 The males of *P. palestinensis* and *P. vesuvianus* are not known.

Distribution and Bionomics of Sandflies in Catania and District.

Sandflies make their appearance in Catania towards the end of May or the beginning of June. In 1930 the sandfly season terminated about October 27. *P. papatasi* and *P. perniciosus* were the first species to appear, and about a week later the other three species, *P. major*, *P. sergenti* and *P. parroti* var. *italicus* were found. The comparative rarity of the three latter species makes it difficult to determine the exact date of their appearance. All the species are commonest at the periphery of the town in the vicinity of stables and gardens and very rare in the centre where there are no suitable breeding places. *P. papatasi* and *P. perniciosus* are the two commonest sandflies; they are constantly present, but their relative frequency varies during the sandfly season. The distribution of these two species is not identical. While *P. papatasi* is present throughout the whole sandfly area, the distribution of *P. perniciosus* is irregular. In the same street one house may be infected with *P. perniciosus* and another entirely free, but all houses in the sandfly belt contain *P. papatasi*. In forming an estimate of the relative frequency of these two species in a given locality the time of collecting and the structure of the house must be taken into account. From 7 a.m. to 11 a.m. all or nearly all the females of both species which fed in a room during the last 12 hours could be caught. In the afternoon most of the *P. papatasi* were still to be found in the room, while practically all the *P. perniciosus* were hidden in cracks in the same room or had left it for other suitable hiding places. In peasants' houses where the walls are irregular and contain deep cracks and the ceiling is made of disconnected beams or loose branches, *P. perniciosus* is difficult to find except in the evening when it comes out to feed. The following numbers of fed females roughly indicate the relative frequency of *P. perniciosus* during the sandfly season.

Numbers of Fed Females of P. perniciosus Caught.

June	260
July	72
August	130. of these about 100 were caught in the last 10 days of the month.
September	340
October.....	527

The numbers of the table refer only to fed females caught between 7 a.m. and 11 a.m. The relative frequency of *P. perniciosus* can be gauged more accurately from the number of unfed females caught in the evenings. This sandfly begins to enter houses shortly after sunset. The males are the first to appear and are followed after an interval of about half-an-hour by the unfed females. *P. major* enters houses an hour or so after *P. perniciosus*. Owing to the striking difference in the appearance of these two species there is no possibility of confusion between them. *P. papatasi* and *P. sergenti* begin to enter houses 3 or 4 hours after *P. perniciosus*. By arranging the time of collecting at suitable hours during the evening it is possible to obtain a pure catch of unfed females of *P. perniciosus*. A house infected with *P. perniciosus* was selected in a suburb of Catania, and the number of unfed females caught in the evenings was noted during the whole sandfly season. 70 to 80 per cent. of the sandflies caught in this house were *P. perniciosus* and the remainder consisted of *P. papatasi*, *P. major*, *P. sergenti* and a few *P. parroti*. The house contained one living room, which was also a public bar, and two bedrooms. The walls and ceiling were smooth and whitewashed and nearly every sandfly could be observed. Immediately after sunset the living room was illuminated by electric light, which was distinctly visible from a considerable distance and attracted unfed *P. perniciosus* which entered through the widely open door. The females which entered up to 11 p.m. were caught in the evening, and those which fed after this time were caught on the following morning. In the adjoining bedroom, which was nearly always kept dark and where children were sleeping in the early hours of the evening, freshly fed females were caught soon after sunset. Several sandflies were captured while feeding on people out of doors. In a donkey stable in the same house no sandflies could be found, probably because of the many cracks and holes in the walls. The average numbers of unfed females caught in one evening in this house was 12 in June, 4 in July, 12 in the first half of August, 20 in the second half, 50 in the first half of September, 70 in the second half. *P. perniciosus* was found to be most frequent during the first 10 days of October. On October 8 over 300 unfed females were observed in the living room. From the middle of October till October 25 the number of unfed *P. perniciosus* fell off rapidly from 20 per evening on the 15th to 3 on the 25th.

The number of *P. perniciosus* found in different houses is variable. The maximum number found was in a Kala Azar house in Centuripe, 733 metres above sea level. During the second half of September an average of about 400 unfed females entered the house each evening. Although *P. perniciosus* is

constantly present throughout the whole sandfly season, there are occasional intervals of a few days during which unfed females are scarce, e.g., from September 10 to September 13 only 7 females per evening were caught, from September 22 to September 24 practically none were observed. The average number of unfed females per evening throughout the second half of September was 50 to 70. On rainy or windy evenings *P. perniciosus* does not enter houses.

P. perniciosus is limited in its distribution by altitude.

It was not found in Cesaro and Enna 1,100 metres above sea level, but was common in Centuripe 733 metres above sea level. *P. papatasi*, on the other hand, is numerous in both Enna and Cesaro.

Feeding Habits.—Laboratory bred *P. perniciosus* does not feed until 3 or 4 days after hatching. This probably also applies to wild insects. *P. perniciosus* immediately after hatching has a large reserve of fat which disappears in 2 or 3 days. The absence of a fat reserve in wild *P. perniciosus* which come out to feed indicates that they are at least two days old. Before feeding the sandflies are rather phototropic, and on emerging from their hiding places make their way towards conveniently lighted rooms. Some of the insects do not enter houses but feed on human beings and possibly animals out of doors. Having entered a house the females do not necessarily feed at once; some of the insects sit on walls for hours before feeding.

The act of feeding is divided into two parts, a preparatory interval during which the skin is pierced and the mouth parts adjusted, and a second part lasting about 1–2 minutes during which blood enters the food canal and the insect gradually gorges itself. In *P. perniciosus* the preparatory interval lasts from $\frac{1}{2}$ to $2\frac{1}{2}$ minutes. In one instance a female was observed for 15 minutes with its proboscis in the skin without drawing blood. In the sandflies of the *Major* group, *P. perniciosus*, *P. perniciosus* var. *tobbi*, *P. major* var. *syriacus* and *P. major*, contractions of the dilator muscles of the pharynx and buccal cavity and the consequent ingestion of fluid do not necessarily follow as a reflex after the activity of the piercing parts. It is possible to keep these sandflies in the Hertig apparatus for half-an-hour, during which time the piercing organs are in constant activity, though fluid seldom enters the food canal. On the other hand, in *P. papatasi* the act of piercing is invariably followed by contractions of the muscles of pharynx and buccal cavity and by the ingestion of fluid. Once the mouth parts are fixed in the skin, *P. perniciosus* is not easily disturbed and can be crushed without difficulty by adults. The amount of blood ingested during a single full feed is variable, the average for *P. perniciosus* being 0.165

emm. This was determined by dissecting out the midgut of recently fed wild insects, emulsifying the total blood meal in 0.1 c.c. of citrate and counting the number of red cells in 1 emm. of the emulsion in a hæmacytometer. Laboratory bred *P. perniciosus* are usually larger than wild ones and take a correspondingly larger feed. The time of feeding for *P. perniciosus* under natural conditions occurs between half-an-hour after sunset and the early hours of the morning. About half the females enter houses and feed after 11 p.m.; the number of unfed females caught in a suitable house between 8 and 11 p.m. is roughly equal to the number of fed females caught in the same house at 7 a.m. next morning. After feeding, *P. perniciosus* hides in dark corners. Unless the room in which it has fed is suitably dark the insects enter cracks in the walls or roof after daybreak, but if light is excluded they remain until noon. Very few females remain in the room 24 hours after feeding. A few sandflies whose ovaries show that they have fed 2 or 3 days previously are occasionally found in houses but they are exceptional.

No females of *P. perniciosus* whose ovaries show development of 2 or more days after the last feed were found to contain fresh blood. It seems that this sandfly does not re-feed until it has laid a batch of eggs. The evidence for this is indirect but conclusive. Recently gorged sandflies having 1-4 ripe eggs in the abdomen were found, and since the number of eggs developed in *P. perniciosus* varies from 14 to 70, the inference is that most of the eggs were laid before the insects re-fed. *P. papatasi* re-feeds every 2-3 days irrespective of the conditions of the ovaries.

P. perniciosus was observed to feed on mammals only. No specimens out of 751 dissected were found with nucleated red cells in the stomach. It prefers mules and cows to man, and the latter to hamsters and mice. If a stable adjoins a sleeping room, the majority of recently gorged females are found in the stable.

Unfed *P. perniciosus* lives up to 5 days in the laboratory. Only from 0 to 15 per cent. of the laboratory bred females fed, although they were offered a feed daily during the whole of their life. In the case of wild unfed females up to 60 per cent. feed if they are offered a feed on the evening of their capture. If they are not allowed to feed on the same evening the majority do not feed at all. In the case of *P. papatasi*, 30 per cent. of females feed on the first day, 50 per cent. on the second day after hatching, 10 per cent. on the third day, 5 per cent. after the third day, and 5 per cent. do not feed at all. If they are not allowed to feed one day, they feed more avidly on the following day.

Breeding.—Breeding was carried out in small porous earthenware vessels.

The vessels were placed on soft earth and the moisture was adjusted empirically by adding water to the earth until no more was absorbed. Soon after the first stage larvæ hatched, food (a mixture of one part of dry rabbit fæces and two parts of sieved earth or a mixture of earth and dried blood and rabbit fæces) was added. Once the larvæ hatched there was no difficulty in rearing them up to the imago. Under laboratory conditions some females died without laying eggs and many died after laying only a part of their eggs; very few laid all their eggs. In no case did a sandfly survive after laying all or a part of its eggs in the laboratory. In nature, as previously stated, *P. perniciosus* lays all or nearly all of its eggs and then re-feeds. Roubaud (1927) also found that under laboratory conditions very few females of *P. perniciosus* survive after laying eggs. Under natural conditions *P. perniciosus* was found to enter stables and hide in deep cracks in the walls within 24 hours after a feed. These are probably their breeding places, for young unfed females were found in the stable, and fed females were found entering holes in the same stable. The exact conditions under which all the eggs are laid and the sandflies survive have yet to be determined. The full cycle from egg to imago in *P. perniciosus* lasts about 5 weeks at a temperature of 28° to 30° C. Like all sandflies whose life history has been studied, *P. perniciosus* hibernates as fourth stage larva.

Hibernation starts as early as the end of August. Out of a batch of 30 eggs laid by a single female on August 26, 1930, all developed into fourth stage larvæ. On October 23 they were taken aboard ship and transported to Palestine. From October 27 they were kept in an incubator at a temperature of 30° C. Between November 12, 1930 and December 14, 1930, 28 larvæ pupated and hatched, but two hibernated in spite of the temperature of 30° C. They remained fourth stage larvæ up to March 16, 1931, when they were found dead. In the layings of sandflies caught from September onwards the percentage of hibernating fourth stage larvæ increased up to 90 per cent. in some broods, although they were kept at a temperature of 30° C. for 14 weeks. Some larvæ were kept at 30° C. and others at a room temperature of 5° to 18° C. A temperature of 30° C., the optimum for the development of eggs laid in July, has a deleterious effect on the hibernating larvæ. At this temperature a few pupate, but about a third of pupæ die, and the mortality in the remaining larvæ is from 70 to 90 per cent. On the other hand, hibernating larvæ kept at a temperature of 5° to 18° C. and protected from rain and wind have a mortality of about 10 per cent. only. After 2 months at this temperature some were placed on a temperature of 30° C. and within 10 days a considerable number pupated. Hibernation appears to be a cyclical phenomenon appearing every three or

four generations even when the eggs and all the larval stages are kept at temperatures approaching 30° C. Whatever its origin, it is no longer merely an adaptation to low temperatures but an essential part of the life cycle of *P. perniciosus*. The hibernation of *P. perniciosus* is in many respects similar to that of *P. papatasi* as determined by Roubaud, who assumed that at low temperatures the hibernating larvæ whose rate of metabolism is low are able to excrete the excess of urates.

Roubaud (1927), working with *P. perniciosus* from Tunis, found that there was no hibernation at a temperature of 30° C. It remains to be determined whether this is due to climatic factors, to racial differences involving differences in metabolism between the sandflies of Tunis and Sicily, or to some other causes. It is to be noted that, contrary to our experience with *P. perniciosus* from Catania, Roubaud found that the Tunisian specimens feed readily on laboratory animals.

The relatively small number of *P. perniciosus* caught in June, as compared to the large numbers of sandflies observed in September and October, indicates a large mortality among the hibernating larvæ under natural conditions. The scarcity of *P. perniciosus* in July, when the insect is much rarer than in June, shows that there is a high mortality among the eggs or young larvæ during the latter month.

Meteorological Conditions in relation to Sandflies.—The rainfall in Catania averages 638 mm. per annum, of which 3 per cent. falls during the sandfly season. When sandflies first appear the medium day temperature is about 20° C. From the middle of July to the end of August, when the conditions for *P. perniciosus* were favourable for completing the full cycle from egg to imago, the medium temperature in the shade varies from 29° to 30° C., the maximum from 29·5° to 34° C., the minimum being almost constantly at 24° C. During June, when the mortality of the eggs or young larvæ is high, the conditions appear to differ from the natural optimum only in temperature, the medium day temperature being 24° to 26° C., the maximum varying from 28° to 30° C., and the minimum from 19·2° to 20·4° C. The relative humidity is fairly constant throughout the whole sandfly season. Climatic conditions are not sufficient to account for the phenomenon of hibernation in *P. perniciosus*. Fourth stage larvæ hibernate in the laboratory where they are protected from the action of rain and kept at constant temperature and humidity. During September, when a large percentage of fourth stage larvæ hibernate, the temperature is about the same or a little higher than in June when no hibernation takes place. During the period of hibernation the surviving larvæ experience a

changing temperature which falls to 3.4°C . (the lowest in February) and which varies from 3.4° to 27.3°C . Raising the temperature alone is not sufficient to prevent or change the phenomenon, which persists for months in the incubator at a temperature of 30°C .

P. major.

This species is present throughout the whole sandfly season, but is commonest in June and July. Of a total of 222 gorged females, 154 were caught in June, 53 in July and 15 in the remainder of the season. Its distribution in Catania is very irregular. It is rare in most parts of the town and common in two quarters (Nesima and Via Palermo). In Nesima it is the commonest of all sandflies at the end of June and beginning of July. During this period 50-100 unfed females could be seen in a room every evening. No specimens were observed in Centuripe where *P. perniciosus* is common. *P. major* enters houses a few hours after sunset. A few females were observed to feed out of doors. As in the case of *P. perniciosus*, the females follow the males by a short interval. The unfed female is the most phototropic of all sandflies and settles in large numbers round lamps and occasionally even on electric torches. Almost immediately after feeding the female becomes extremely photophobic and hides in the nearest dark corner. This sandfly is very difficult to manipulate in the laboratory. Breeding experiments gave very poor results and the majority of females refused to lay eggs in the laboratory. Very few unfed wild females fed in the laboratory, although they feed readily under natural conditions.

P. sergenti.

Out of a total of 100 gorged females, 62 were caught in July. The distribution of this sandfly in Catania is irregular. It is common in a few streets only. The bionomics of this species have been discussed elsewhere.

P. parroti var. *italicus*.

Only 20 females were caught. No particular attention was paid to the bionomics of this species which feeds on lizards and, as far as our observations during 1929 and 1930 go, never on man.

616.929.3 + 591.67: 595.771 *Phlebotomus*.

Investigations on Mediterranean Kala Azar. IV.—Infection of Sandflies with Leishmania infantum. Natural Parasites of Wild Sandflies in Catania. Observations on Trypanosoma ptyodactyli Catonillard.

By S. ADLER and O. THEODOR.

(Kala Azar Commission of the Royal Society.)

(Communicated by H. H. Dale, Sec. R.S.—Received May 7, 1931.)

Three methods of infecting sandflies with *L. infantum* were employed :—

- (1) Feeding on cultures or on infected bone marrow through sterile membranes of skin. The method has been previously described.
- (2) Feeding on human cases.
- (3) Feeding on infected hamsters.

The first method has the advantage that the results have a quantitative as well as a qualitative significance. The behaviour of cultures of *L. infantum* (from Italy) in *P. papatasi* has already been recorded (1931). Further work was carried out with infected bone marrow. In Professor Longo's clinic in Catania puncture of the spleen is avoided and the diagnosis of *L. infantum* in children is established by examining the bone marrow. The top of the tibia is punctured with a special needle, a few drops of bone marrow are removed and smears made. In some cases bone marrow (from 0.5 to 2 c.c.) was drawn into a syringe, and 0.5 c.c. were placed in the feeding apparatus into which sandflies had been previously introduced. *P. papatasi* feeds readily through membranes, while *P. sergenti* and *P. major* feed rarely and *P. perniciosus* does not feed at all by this method.

In 11 experiments carried out between June 4, 1930, and July 26, 1930, a total of 152 females of *P. papatasi* fed on infected human bone marrow. The sandflies were dissected at intervals of 1 to 7 days after the feed. In six experiments in which 10, 10, 3, 33, 1 and 12 sandflies fed, none were positive. In these experiments the smears of bone marrow showed L.-D. bodies from 1 per field to 1 per 40 fields (magn. 900 ×). In five other experiments the numbers of sandflies positive were 3 out of 11, 1 out of 4, 3 out of 14, 4 out of 39, and 2 out of 13. In these experiments the concentrations of parasites

varied from 1 in 20 fields to 7 to a field. Even in the latter concentration the infection rate was only 3 out of 11. It is assumed that the L.-D. bodies in the bone marrow correspond in every way to those in the circulating blood, for there is no evidence to the contrary and cultures made from the bone marrow resemble those taken from the blood in all details except that in the former, the flagellates appear earlier in culture because of the larger number of parasites inseminated. The positive sandflies were dissected from 2 to 6 days after the infecting feed. The results of the dissection are shown in Table I.

Table I.—Feeding of *P. papatasi* on Bone Marrow.

Sandfly No.	Date of feed.	Date dissected.	Result.
13	7.6.30	10.6.30	Long motionless flagellates in stomach.
18	7.6.30	12.6.30	Heavy infection. Short flagellates attached in cardia. Long flagellates free in stomach.
21	7.6.30	13.6.30	Heavy infection. Mass of long free flagellates in lower part of cardia. A few attached in upper part. A few free in stomach.
26	12.6.30	16.6.30	Heavy infection. Many free parasites from middle of cardia to the stomach. Few in hindgut.
60	3.7.30	6.7.30	Moderate infection. Long flagellates attached in cardia and free in stomach.
61	3.7.30	6.7.30	Heavy infection of long flagellates in stomach. A few attached in cardia.
62	3.7.30	7.7.30	Few long flagellates in stomach, many in cardia.
105	5.7.30	9.7.30	Slight infection of long flagellates in cardia and stomach, few attached in cardia.
113	5.7.30	10.7.30	Moderate infection in stomach, a mass of long flagellates attached in cardia.
122	5.7.30	10.7.30	Mass of long flagellates attached in cardia. A few free forms in stomach.
125	5.7.30	11.7.30	Slight infection. A few long flagellates attached to top of cardia. A few free forms in stomach.
127	9.7.30	11.7.30	Many long flagellates attached in cardia. Few free in stomach.
128	9.7.30	11.7.30	Few free flagellates in stomach.

Table II.—Feeding Experiments on Human Beings.

Case No.	Date of feed.	Blood culture.	No. of sandflies fed.		No. positive.	Remarks.
			Papa- nae	Perni- ciae		
42	2.7.30	+ with 30 cm.	11			Fed on defibrinated blood through a membrane. Dissected after 3-9 days. All negative.
35*	5.7.30	+ with 30 cm.	13			Dissected after 2-5 days. All negative. Relapse case tartar emetic resistant.
35	9.7.30	+ with 30 cm.				Dissected after 1-6 days. All negative.
44*	10.7.30	Negative	34			Dissected after 1-14 days. All negative. Relapse case tartar emetic resistant.
67	7.8.30	+ with 30 cm.				Dissected after 2-8 days. All negative. 2 <i>P. sergenti</i> dissected after 7-8 days also negative.
69	12.8.30	+ with 2 cm.				Dissected after 4-8 days. All negative.
69	14.8.30	+ with	10			Dissected after 5-6 days. 1 <i>P. sergenti</i> dissected after 5 days, negative.
69	15.8.30	+ with 2 cm.	10			Dissected after 4-7 days. All negative.
78	5.9.30	Not taken		28		Dissected after 2-9 days. All negative.
78	10.9.30	Not taken				Dissected after 2-6 days. One after 4 days positive. Slight infection. Flagellates attached to top of cardia.
78	16.9.30	Not taken				Dissected after 4 days. One positive. Moderate infection. Long flagellates mostly in cardia. Sandflies fed 1 week after injection of tartar emetic.
86	19.9.30	Not taken				Dissected after 4-5 days. All negative.
88	20.9.30	Not taken				Dissected after 4 days. All negative.
88	23.9.30	Not taken			1	Dissected after 3-4 days. In one slight infection. Long forms in cardia, none in stomach.
88	26.9.30	Not taken		5 1 major	—	Dissected after 5 days. All negative.

* In cases which relapse after treatment with tartar emetic, parasites in the spleen and bone marrow are usually very scanty.

Table II.—continued.

Case No.	Date of feed.	Blood culture.	No. of sandflies fed.		No. positive.	Remarks.
			Papatasii.	Perniciosus.		
90	1.10.30	Not taken				One dissected after 6 days, positive. Heavy infection of long forms in stomach and cardia. Many attached in cardia. Bacteria in midgut which were probably the cause of death. The midgut was kept in saline for 6 hours during which the flagellates were completely lysed.

Feeding Experiments on Hamsters.

The experiments were carried out between June 18, 1930, and October 22, 1930. The fed sandflies were kept at laboratory temperature. The *P. papatasii* used in the experiments were mainly laboratory bred. The *P. perniciosus* were mainly wild and unfed and a few were laboratory bred. Wild sandflies had to be used because only few laboratory bred *P. perniciosus* fed on hamsters. The *P. major* and *P. sergenti* were all wild specimens without blood in their stomach.

Two Chinese hamsters were used for the experiments, one inoculated with cultures (hamster No. 1) and the other with bone marrow and cultures (hamster No. 2).

P. papatasii.—One hundred and ninety-seven sandflies fed on hamster No. 1 and were dissected from 1 to 8 days after feeding. Of these seven dissected from 3 to 8 days after the infecting feed were positive, i.e., the infection rate was 3·6 per cent. Forty sandflies fed on hamster No. 2. All were negative.

P. sergenti.—Eleven sandflies fed on hamster No. 1 and were dissected from 1 to 6 days after feeding. All were negative.

P. perniciosus.—Eight sandflies fed on hamster No. 2 and were dissected from 2 to 4 days after the feed. All were negative.

Three hundred and forty-one fed on hamster No. 1 and were dissected from 1 to 13 days after the feed. Three hundred and thirty sandflies were found positive, i.e., an infection rate of 96·8 per cent.

The behaviour of *L. infantum* in *P. perniciosus* was studied by fresh dissections and serial sections of whole sandflies or their heads. The sandflies

Table III.—Infection of *P. papatasi* on Hamster No. 1.

Sandfly No.	Date of feed.	Date dissected.	Temperature during experiment.	Result.
122	31.7.30	4.8.30	27–29° C.	Heavy infection from top of cardia to hindgut. Long flagellates, many attached to cardia.
178	11.8.30	16.8.30	27–30° C.	Heavy infection. Cardia choked with flagellates.
181	11.8.30	16.8.30	27–30° C.	Moderate infection in stomach and cardia. Flagellates nearly all free. Medium and short forms.
425	13.9.30	16.9.30	25–30° C.	Heavy infection in stomach, cardia and oesophageal diverticulum.
696	9.10.30	17.10.30	18–24° C.	Infection in cardia, stomach and hindgut.
739	16.10.30	24.10.30	18–24° C.	Heavy infection. Cardia choked with flagellates, mainly long forms. Some short forms present in cardia.
740	16.10.30	24.10.30	18–24° C.	Heavy infection. Cardia choked with long flagellates.

used for sectioning were fixed in 70 per cent. alcohol. In freshly dissected specimens it is usually impossible to study the flagellates in the buccal cavity and pharynx, unless these are dissected out of the head. The description of the behaviour of flagellates in these parts is therefore based on serial sections stained with Mayer's hæmalum or Heidenhain's iron hæmatoxylin.

In the laboratory the infected sandflies were kept at a temperature of 22° to 30.5° C. At a temperature of 27° to 30° C. there was already a heavy infection in the midgut and cardia after 2 days. There were many long flagellates in the cardia and some were attached to the rhabdorium. Three days after feeding the oesophagus was also infected. In the majority of the freshly dissected sandflies the infection was found in the midgut and the oesophagus, but when the infection was very heavy the hindgut and rectum were also swarming with long flagellates.

Types of Flagellates.

Both in *P. perniciosus* and *P. major* infected on hamsters, and in *P. papatasi* infected on human bone marrow or hamsters, the following types of flagellates were seen :—

- (1) Short, thick flagellates 8 to 11 μ long and about 5 μ wide with the flagellum rudimentary or relatively long. These are often seen in division (fig. 1, *a-c*).
- (2) Flagellates from 13 to 21 μ long and up to 2.5 μ wide. This type also has many dividing forms (fig. 1, *d-f*).
- (3) Long flagellates from 22 to 30.5 μ long and 0.8 to 1.5 μ broad. The flagellum is usually about half the length of the body or less, but some are found in which the flagellum is as long as the body. In sandflies dissected 2 to 6 days after a feed these forms are the most numerous (fig. 1, *g-i*).
- (4) Very short flagellates from 4.7 to 10 μ in length and up to 1 μ broad. The flagellum is usually longer than the body (fig. 1, *k-r*).

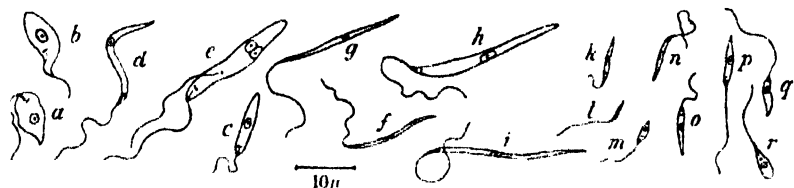


FIG. 1.—Developmental forms of *L. infantum* in sandflies. *a-c*, type 1; *d-f*, type 2; *g-i*, type 3; *k-r*, type 4; *k-o*, from cardia of sandflies 4–12 days' development; *p-r*, from proboscis of living sandflies in Hertig apparatus.

These forms (4) are typical of late infections in *P. perniciosus*. They appear as early as 4 days after the infecting feed at 27° to 30° C. and after 6 days at temperatures less than 25° C. These small forms first appear in the upper part of the cardia in the neighbourhood of the œsophageal valve. Their small size, their position and their behaviour in the freshly dissected sandfly are very characteristic. They have a very marked tendency to move anteriorly in the sandfly and often swim out from a choked cardia in large numbers before the stomach is subjected to the pressure of the coverslip. In freshly dissected specimens they are found only in the cardia, particularly in the upper part, and never in the stomach. It was not possible to trace transition forms between these flagellates and others, although in the late stages of infection in the sandfly there is a tendency for all the flagellates to be shorter than in the first 4 to 6 days of the infection. It should be noted that the small flagellates do not appear in all the infected sandflies. As in the case of *L. tropica* in *P. papatasi*, several sandflies were found in which the cardia was choked with flagellates, although the stomach contained few or none.

It is evident from the above description that in *P. perniciosus*, *L. infantum*

undergoes a definite cycle in which fairly definite morphological forms follow in regular order. The short forms with long flagella are the end point of this cycle in the sandfly, and under natural conditions these are the most likely to enter a vertebrate host. It remains to be determined whether they are more infective than the other types of flagellates.

Course of Infection in the Chitinous Parts.

The sections show that :—

- (1) After 3 days at a temperature of 27° to 30° C. the pharynx may be packed with flagellates.
- (2) After 4 days the posterior part of the buccal cavity is affected.
- (3) After 5 days the infection may extend up to the anterior end of the buccal cavity.

At lower temperatures (22·5° to 26·5° C.) the whole of the pharynx may be invaded after 4 days and the buccal cavity after 5 days. There are slight differences in individual sandflies and in some the buccal cavity is not invaded even after 5 days.

The flagellates may fill the whole lumen of the pharynx but in the buccal cavity they are usually confined to the roof, which is formed by the junction of the two lateral plates.

The proboscis is invaded after the fifth day and flagellates may reach almost to the tip of the proboscis on the sixth day. They are confined entirely to the inferior surface of the epipharynx. Large numbers of flagellates may lie along the whole inferior surface of the epipharynx or individuals and small groups may be disposed at irregular intervals. Flagellates of types 3 and 4 are found in the pharynx; in the buccal cavity and proboscis only type 4 is found.

Although in general outlines the development of *L. infantum* in *P. perniciosus* is similar to that of *L. donovani* in *P. argentipes* and *P. chinensis*, there are some differences in details. In no other sandfly have such large flagellates been observed. Owing to the kindness of Dr. E. Hindle, smears of *L. donovani* from *P. chinensis* were examined and the largest forms found were 23 μ . In *P. papatasi* flagellates of *L. tropica* attain the size of 27 μ .

The rate of development is more rapid in *P. perniciosus* than in *P. argentipes* or *P. chinensis*. In the latter flagellates reach the pharynx and buccal cavity after 6 days. In *P. argentipes*, according to Shortt (1929), the flagellates reach the junction of pharynx and buccal cavity on the seventh day and the anterior

end of the buccal cavity on the eighth to tenth day. The most anteriorly placed flagellates may project into the prestomium. In *P. perniciosus* flagellates reach the tip of the epipharynx and are found among its terminal teeth as early as the sixth day (Plate 18, fig. 5). When flagellates occur among the terminal teeth of the epipharynx their entry into the skin during the act of biting appears inevitable.

It remains to be determined whether the more rapid rate of development in *P. perniciosus* is due to some peculiarity in the sandfly or in the parasite.

The Exit of L. infantum through the Proboscis of P. perniciosus.

In order to study the possible mode of infection it was necessary to resort to Hertig's method of feeding through capillaries, as none of the sandflies re-fed on hamsters. By this method the biting parts of the sandfly are manipulated into the opening of a fine capillary of sufficiently small bore to exclude the labium. A little citrate (about 2 cmm.) was blown down the capillary after the mouth parts were in position. Under these conditions *P. papatasi* feeds very readily, but *P. perniciosus* seldom ingests fluid, although the piercing parts are active.

Fifteen sandflies from 5 to 13 days after the infecting feed were used for the experiment. After an interval from 1 to 3 minutes the sandflies were removed, the total amount of fluid in the capillary was blown on to a slide and examined under a coverslip. In six cases the fluid was found to contain flagellates. The number of flagellates seen in the different experiments were 1, 1, 1, 4, 20, and several hundreds. In the last case flagellates were sufficiently numerous for making a stained preparation. The flagellates (fig. 1, *p-r*) were mainly from 6 to 11 μ in length with the flagellum longer than the body. One flagellate 17.4 μ long was found. In contrast to the flagellates from the midgut or from cultures, those recovered from the capillary were sluggish and the majority were inactive in spite of the fact that the flagellum was intact. (One single aflagellar form was found in a stained preparation. The flagellum in this case may have been broken off while the smear was made.) None showed a translatory movement. (The movement of a number of active flagellates from cultures was measured and found to be up to 20 μ per second.) It is therefore evident that the flagellates underwent profound changes in the proboscis. It remains to be determined experimentally whether flagellates from the proboscis are infective for man or animals.

The above experiments show that *L. infantum* can leave the proboscis of infected *P. perniciosus* and enter a fluid medium during the act of piercing,

but the mechanism by which the flagellates leave the proboscis is not yet clear. It does not appear probable that the flagellates leave the proboscis by their own activity, for they show practically no translatory movements in the fluid. Flagellates from the midgut or from cultures remain active for hours in citrate.

Sections of the sandflies which gave no flagellates in the Hertig apparatus showed that infections of the pharynx and buccal cavity are not in themselves sufficient to cause the exit of flagellates under the conditions of the experiment. It still remains to be determined to what level the flagellates must descend before they can leave the proboscis. In one case where 20 flagellates were recovered from the capillary a section showed that at least another 20 flagellates remained in the proboscis, of which some were only 100 μ from the distal end of the epipharynx.

The exit of flagellates from the proboscis of *P. perniciosus* is suggestive. It is in accord with the most distinctive feature of Mediterranean Kala Azar, i.e., its relative frequency in infants under 12 months who cannot crush insects.

P. major.—Out of 13 sandflies which fed on the hamster 12, i.e., 92.3 per cent., were positive. The infections were very heavy and resembled those in *P. perniciosus*. The flagellates ascended the cardia and attached themselves as early as 2 days after the feed. Owing to the relatively small number of sandflies which fed it was not possible to make as extensive a study as in the case of *P. perniciosus*.

Discussion of Feeding Experiments on Hamsters.

No L.-D. bodies were found in blood smears of hamster No. 1 made at the time the feeding experiments were carried out, but sections of the skin showed that the corium contained infected macrophages.

The high infection rates in *P. perniciosus* and *P. major* show that almost every sandfly ingested L.-D. bodies (hamster No. 1 received the first injection of *L. infantum* from cultures on May 13, 1930, and one *P. perniciosus* which fed on June 18, 1930, was infected. The corium or the blood must, therefore, have contained L.-D. bodies within 5 weeks after the first inoculation of *L. infantum*.) It is therefore concluded that almost all the specimens of *P. papatasi* and *P. sergenti* which fed on the hamster also ingested L.-D. bodies, but that these two sandflies are not suitable for the development of *L. infantum*. In the case of *P. papatasi*, 6 out of 7 of the positive sandflies which became infected on hamster No. 1 fed after August 11, 1930. Up to that time the infection rate in *P. papatasi* was 1 out of 134. During this period the infection

rate in *P. perniciosus* was 83·3 per cent. The heavier infection rate in *P. papatasi* after August 11, 1930 (9·5 per cent.) was obviously due to the increasing number of L.-D. bodies in the corium. The comparative infection rate up to August 11, 1930, 83·3 per cent. in *P. perniciosus* to 0·75 per cent. in *P. papatasi*, is a striking illustration of the relative adaptability of *L. infantum* in these two sandflies.

The high infection rate produced in *P. perniciosus* and *P. major* cannot be regarded as an accident, and is considered to show a specific host-parasite relationship between *L. infantum* and these two sandflies. This specificity still remains to be investigated. It is probably not due to differences in the p_H of the intestinal juices of the different sandflies, for in cultures *L. tropica* and *L. infantum* have the same range of p_H and every known medium suitable for the development of *L. tropica* is equally suitable for the development of *L. infantum*. Nevertheless, Palestinian strains of *L. tropica* produce a high infection rate in *P. papatasi* and Italian strains of *L. infantum* a low one.

Experiments with L. infantum from artificially infected P. perniciosus.

Chinese hamster No. 4.

14.10.30. Inoculated intraperitoneally with *L. infantum* from the mid-gut of sandflies Nos. 678–680. These sandflies were infected by feeding on Chinese hamster No. 1 on 9.10.30. In sections of the head Nos. 678 and 679 showed infections in the pharynx and No. 680 was also infected in the buccal cavity.

Result: The animal died 9.1.30 and was found to have visceral *Leishmaniasis*.

White mice.

25.9.30. Five mice inoculated subcutaneously with *L. infantum* from six *P. perniciosus* which had been infected 5 days previously by feeding on Chinese hamster No. 1. The infection was very heavy in all the sandflies.

Result: Negative during an observation period of 3 to 6 months.

2.10.30 to 18.10.30. Seven mice were inoculated intraperitoneally with *L. infantum* from 13 heavily infected sandflies from 5 to 10 days after feeding on Chinese hamster No. 1.

Result: Negative after an observation period of 6 months.

Experiment on man.

16.10.30. Whole midguts of sandflies Nos. 669 and 670 inoculated into

two points on the leg of a human being. Sandflies Nos. 669 and 670 were infected by feeding on Chinese hamster No. 1 (5.10.30). 16.10.30. No. 669 was found heavily infected from proboscis to stomach. There were many short forms in the cardia.

16.10.30. No. 670 gave a negative result in the Hertig apparatus. On dissection a heavy infection was found in the midgut. There were many short forms in the cardia. In section the pharynx was found choked with flagellates and the buccal cavity was also infected. The volunteer had been previously infected with *L. tropica* from flagellates of a naturally infected *P. papatasi*. This infection healed spontaneously after 3½ years and further attempts to re-infect with *L. tropica* gave negative results. Inoculations with flagellates of *L. tropica* dead or living always gave rise to a typical skin reaction—hyperæmia after a few days and scaling.

Result: After 2 weeks two scaling papules appeared on the site of inoculation. These still persist (31.3.31) but repeated smears failed to reveal the presence of L.-D. bodies.

Experiment on a dog.

30.10.30. Midgut of sandflies No. 747 and No. 753 inoculated into both ears of a dog. Sandfly No. 747 was infected by feeding on Chinese hamster No. 1 on 21.10.30 in Catania, and was transported to London (during part of the journey it was subjected to a temperature of 5° C.) where it was found dead. On dissection many active flagellates were found in cardia and stomach. Sandfly No. 753 was infected by feeding on Chinese hamster No. 1 on 22.10.30 in Catania and was transported to London, where it was found dead. On dissection numerous active flagellates were found in stomach and cardia.

Result: 5.5.31. Dr. C. M. Wenyon kindly examined smears of bone marrow and spleen and found them negative.

Natural Parasites of Wild Sandflies in Catania.

Out of 3839 ♀♀ of *P. papatasi*, 100 of *P. sergenti* and 222 of *P. major*, none were found infected with *Leishmania*. Out of 751 ♀♀ of *P. perniciosus* one caught in a Kala Azar house in Centuripe in a room where a patient was sleeping was found to be infected (5.10.30). The infection was slight; about 20 flagellates were found, of which some were attached in the cardia.

5.10.30. The whole midgut was rubbed into a scarified point on the hindleg of a Chinese hamster.

26.1.31. Partial splenectomy. Spleen smears negative.

The above numbers refer only to fed or partially fed females caught in or near Kala Azar houses and dissected 1 to 5 days after capture. They do not include sandflies used for breeding.

One female of *P. parroti* var. *italicus* was found infected with *Crithidia*, which were probably stages of *Trypanosoma ptyodactyli*. They will be described below.

A few females of *P. papatasi* were found infected with fungi in the alimentary tract. Nematodes were found in the coelome in five sandflies and others were found infected with ectoparasitic mites (from 2 to 11).

A few specimens of *P. perniciosus* were found infected with fungi and yeasts in the coelome.

Observations on Trypanosoma ptyodactyli Catouillard.

This trypanosome was found in the blood of 17 out of 49 specimens of *Tarentola mauritanica*. It grows readily on Locke Serum or Locke Blood Agar. Cultures live up to 2 months, but it is advisable to subculture every 2 or 3 weeks. In culture the organism is very pleomorphic and all forms occur between aflagellar bodies 5 μ in diameter to *Crithidia* 30 μ long (fig. 2, *a-e*). Trypanosomes also occur (fig. 2, *h-i*). In some primary cultures the majority of the organisms may be aflagellar. After a number of generations in culture the organism may lose its flagellum or develop only a rudimentary



FIG. 2.—Culture forms of *T. ptyodactyli*. *a-b*, aflagellar forms; *c-e*, *Crithidia*; *f-g*, piriform bodies in old cultures; *h-i*, trypanosomes.

one and produce rich cultures of piriform bodies without a flagellum (fig. 2, *f-g*).

A Trypanosome found in a wild P. parroti var. italicus.

The forms in the midgut were mainly *Crithidia* but a few trypanosomes were also found. The *Crithidia* varied in size from 6, 7 μ to 30 μ and the flagellum was either free or very short. The trypanosomes were up to 16 μ long (fig. 3).

The infection was rather heavy and many flagellates were found in the cardia. A number of flagellates were attached to the rhabdiorium. Sections of the head showed the infection to extend as far as the anterior part of the pharynx.

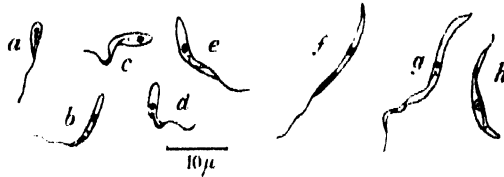


FIG. 3.—Trypanosomes found in naturally infected *P. parroti*. *a-e*, short *Crithidia*; *f-g*, long *Crithidia*; *h*, trypanosome.

Infection of Sandflies with T. ptyodactyli.

Fourteen females of *P. papatasi*, laboratory bred, fed on a naturally infected gecko and were dissected 2 to 12 days after feeding. Ten were found to be infected. The infections were heavy and the flagellates were confined to stomach and cardia and did not descend into the hindgut. In all cases flagellates were attached to the rhabdiorium of the cardia.

Three wild *P. parroti* fed on the same gecko and were dissected 4 days later. All were heavily infected in cardia and stomach. In one sandfly sections showed the pharynx, œsophagus and midgut choked with flagellates but the hindgut was not infected. The type of flagellates found in *P. papatasi* and *P. parroti* resembled those found in the wild *P. parroti*.

Thirty mites collected from infected geckoes were examined and found free from flagellates.

Only a small number of flagellates are necessary to infect a sandfly, for no trypanosomes were found in blood smears of the gecko used for the experiment.

The trypanosome in the sandfly clearly has an anterior position. The infection persists for at least 9 days in *P. papatasi* and is not affected by refeeding. It is therefore probable that *T. ptyodactyli* is transmitted by the bite of a sandfly. *P. papatasi* is probably not the carrier, for in nature it does not feed on geckoes, although it does so under laboratory conditions if deprived of other food. Out of 3839 *P. papatasi* dissected none were found to contain nucleated red cells in their midgut. On the other hand, *P. parroti* in nature usually feeds on geckoes and is therefore probably the transmitter of *T. ptyodactyli*.

Investigations on Mediterranean Kala Azar. V.—Distribution of Sandflies of the Major Group in Relation to Mediterranean Kala Azar. Summary and Conclusions. References.

By S. ADLER and O. THEODOR.

(Kala Azar Commission of the Royal Society.)

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The work of the Indian Kala Azar Commission, the Royal Society Commission in China and the observations recorded above indicate that the visceral *Leishmanias* are particularly adapted to sandflies of the *Major* group, i.e., *P. argentipes* in India, *P. chinensis* in China, *P. perniciosus* and *P. major* in Sicily. Parrot, Donatien and Lestoequard (1930) found that *P. perniciosus* which fed on a dog with visceral *Leishmaniasis* and skin lesions became infected. The infection was confined to the stomach of the sandflies 3 days after the infecting feed. The above sandflies have all been infected by feeding on experimental animals and the first three have also been infected on man. Natural infections with *Leishmania* have been recorded in *P. argentipes* and *P. perniciosus*. *P. perniciosus* var. *tobbi* and *P. major* var. *syriacus* have been infected by feeding on cultures of *L. infantum*.

It is therefore of interest to consider the known distribution of sandflies of the *Major* group in relation to visceral *Leishmaniasis* in the Mediterranean, for the two species not included in this group which feed on man, i.e., *P. papatasi* and *P. sergenti*, have been excluded experimentally as possible carriers.

P. perniciosus.—This species is present in endemic foci in Italy, France, Spain, Tunis and Algiers. In Italy the distribution of this sandfly coincides with that of the disease. The absence of this sandfly and the disease at high altitudes in the neighbourhood of endemic foci is significant. In Sicily, Kala Azar is mainly a disease of the coastal plain. The largest endemic foci in Italy, i.e., Naples, Palermo, Catania are all on or near the coast. In these foci there are districts of maximum intensity on the outskirts of towns, e.g., Via Fossa Creta, Ognina and Cibali in Catania, Villabate and Aquasanta near Palermo, and Bosco Trecase near Naples. In some houses in these districts *P. perniciosus* is the most common sandfly. In Catania itself both the sandfly and the disease are rare in the centre of the town. In the interior of Sicily

where *P. perniciosus* has not been found, Kala Azar is very rare even in districts where the altitude is less than 700 metres. The climatic conditions in the coastal region of Sicily are obviously more favourable for the development of this sandfly than those of the interior. *P. perniciosus* has not been found in Palestine and Syria where there are few cases and in Greece and Macedonia where there are many cases. It does not, therefore, account for the whole of Mediterranean Kala Azar.

P. major.—The local race of this species is common in several quarters of Catania where the disease is common but where *P. perniciosus* is also abundant. The local distribution of the disease is much wider than that of *P. major*. It is possible that Catania is the heaviest centre of the disease partly because of the presence of this species. In India no relationship has yet been observed between the distribution of *P. major* and that of Kala Azar, but it is not yet known to what extent racial differences in sandflies are accompanied by biological differences in general and suitability for the development of *Leishmania* in particular.

P. perniciosus var. *tobbi*.—This species is present in Rosh Pinah in Palestine, from where the disease has not yet been recorded. It is also present in Resht in Persia.

P. ariasi is present in Valencia, an important endemic focus.

P. macedonicus.—This sandfly particularly requires experimental investigation. In the absence of experimental evidence, and in spite of the lack of a fuller knowledge of the sandflies of Macedonia and Greece, this sandfly may be suspected as a probable vector of Kala Azar because of its close relationship to *P. perniciosus*.

P. vesuvianus is present near Naples in a locality where Kala Azar is abundant and therefore requires further investigation. It is too rare to account for the bulk of the cases in the neighbourhood of Naples.

P. major var. *syriacus* is present in Syria and Palestine and *P. canaaniticus* in Palestine where the disease is rare.

P. chinensis.—One race of *P. chinensis* is present in Macedonia in an endemic focus and is suspect because of the work of Patton and Hindle (1927) and of Young and Hertig (1926) in China. The epidemiology of the disease in Macedonia, however, is very different from that in China. Another similar race of *P. chinensis* has been recorded from North Palestine, where Kala Azar is unknown.

The knowledge of other species of the *Major* group in the Mediterranean basin is too scanty for drawing conclusions.

The Bionomics of Species in Relation to Epidemiology.

As previously pointed out, the epidemiology of the disease must be dependent on the bionomics of the insect vector, on the susceptibility of various groups in the population and on the frequency of parasites in the blood. In the case of *P. perniciosus* the spread of the disease is limited by the following factors :—

(1) The relative scarcity of parasites in the blood. Direct blood cultures show that an average case has at least one parasite, or what is more likely, one infected macrophage per 30 cmm. of blood. Since the average feed of *P. perniciosus* is 0.165 cmm. of blood the infection rate in sandflies feeding on cases may be estimated roughly as 1 per 200, assuming that a single parasitised macrophage gives rise to an infection if ingested by *P. perniciosus*. It was not possible to test this estimate satisfactorily, because in most cases permission to feed sandflies on patients was not obtained, although under natural conditions hundreds of these insects feed on them. Cases where 5 cmm. and 2 cmm. of blood give a positive culture are not rare, but they are not usual. In Indian cases parasites are more numerous in the blood and infection rates of 42 per cent. have been obtained in *P. argentipes*.

(2) An infected *P. perniciosus* after laying its eggs may refeed on a mule, cow or a naturally immune human adult and not necessarily on a susceptible infant.

(3) The seasonal prevalence of the sandfly. In the case of *P. perniciosus* transmission is possible throughout the whole sandfly season, but is most likely from the middle of August to the middle of October when the sandflies are most numerous.

If the sandfly theory is accepted as a working hypothesis, the characters of an endemic centre should provide some clue, even if a negative one, as to the vector. In the Kassala and Blue Nile districts the epidemiology of Kala Azar, in so far as it has been investigated by Bousfield (1911), resembles that of India rather than that of Italy. It can, therefore, be confidently predicted that *P. perniciosus* is not the vector of the disease in the Sudan but some other sandfly of the *Major* group. Up to the present *P. papatasi* is the only anthrophilic sandfly recorded from the Sudan, but further investigations will probably reveal the presence of a species of the *Major* group.

The Behaviour of Leishmanias in Sandflies.

The behaviour of *Leishmanias* from cultures or infected animals has been studied in a number of sandflies and it is now desirable to analyse the various

factors involved. Such an analysis is of practical value, for a few experiments may limit the search for the vector in a given district to one or two species.

The following *Leishmanias* have been studied in sandflies :—

(1) *L. tropica* (Bagdad and Palestinian strains) in *P. papatasi* and *P. sergenti* (Adler and Theodor, 1925 *et seq.*), Algerian strain (Parrot and Donatien, 1927).

(2) *L. ceramodactyli* in *P. papatasi* (Adler and Theodor, 1929).

(3) *L. agamæ* in *P. papatasi* (David, 1929).

(4) *L. donovani* (Chinese strains) in *P. sergenti* var. *mongolensis*, *P. chinensis* and *P. squamirostris* by Young and Hertig (1926). In *P. sergenti* var. *mongolensis* and *P. chinensis* by Patton and Hindle (1927). In *P. papatasi* by Adler and Theodor (1930).

(5) *L. donovani* (Indian strains) in *P. argentipes* by Knowles, Napier and Smith (1924), and the Indian Kala Azar Commission (1924 *et seq.*) in *P. papatasi* by Adler and Theodor (1930) and Napier and Smith (1927).

(6) *L. infantum* (Italian strain) in *P. papatasi*, *P. perniciosus*, *P. major* and *P. sergenti* (Tunis strain) in *P. papatasi*, *L. canina* (visceral strain) in *P. papatasi* by Adler and Theodor (1929 and 1930).

(7) *L. brasiliensis* in *P. papatasi* by Adler and Theodor (1927 and 1930).

The behaviour of the *Leishmanias* of mammals in sandflies may be classified under three heads :—

(1) A relatively small number of L.-D. bodies or flagellates produce infections, the flagellates tend to an anterior position, and the infection in the sandfly persists, *e.g.*, *L. tropica* (Palestinian strain) in *P. papatasi* (Baghdad strain) in *P. sergenti* and *L. infantum* (Italian strains) in *P. perniciosus*.

(2) A large-number of L.-D. bodies or flagellates (more than can be obtained by a sandfly under natural conditions) produce infections in sandflies and the flagellates tend to an anterior position, *e.g.*, *L. donovani* (Indian strains) and *L. infantum* (Italian strains) in *P. papatasi* :—

(a) The infection persists throughout the whole life of the sandfly, *e.g.*, Italian strains of *L. infantum* in *P. papatasi*.

(b) The infection rate diminishes with time, *e.g.*, Indian strains of *L. donovani* in *P. papatasi*.

In the case of *L. infantum* (Italian strains), *P. papatasi* can be practically excluded as a vector, for L.-D. bodies in numbers far larger than those found in natural infections produce a low infection rate.

(3) A large number of flagellates produces an infection which is nearly

always confined to the stomach, e.g., a strain of *L. brasiliensis* in *P. papatasi*, *L. donovani* (Chinese strains) in *P. sergenti* var. *mongolensis*. In the latter case the flagellates disappear after the blood meal has been digested.

In cases where the behaviour is of the latter two types the sandflies concerned can be excluded as vectors, whereas the first type of behaviour indicates that the sandflies concerned are possible vectors. Further studies are required to compare the behaviour of closely related strains of *Leishmania* in sandflies. Baghdad and Palestinian strains of *L. tropica* are indistinguishable serologically, nevertheless they show some points of difference in their behaviour in sandflies. The Palestinian strains produce a high infection rate both in *P. papatasi* and *P. sergenti*. The Baghdad strains produce a high infection rate in *P. sergenti* and a low infection rate in *P. papatasi* (1930). Moreover, in Baghdad and district the main distribution of Oriental sore coincides with that of *P. sergenti* and not with that of *P. papatasi*. Similarly, Chinese and Indian strains of *L. donovani* are biologically very close. Hindle has made an interesting observation which may prove to be of great significance. He found that *P. chinensis* after feeding on a hamster infected with *L. donovani* (Indian strain) became infected, but the flagellates were confined to the stomach, although Chinese strains of *L. donovani* ascended to the buccal cavity.

There is therefore some evidence that strains of *Leishmania* which become particularly adapted to a single species of *Phlebotomus* do not develop well in other sandflies in which closely related strains of *Leishmania* develop readily.

Summary and Conclusions.

The epidemiology of Mediterranean Kala Azar excludes the possibility of transmission by contamination. The rarity of family infections and house to house infections, the rarity of the disease among the poorer urban population in the centre of large cities situated in endemic foci are not compatible with transmission by direct contact or by oral infection. The demonstration of parasites in the circulating blood of 91.7 per cent. of cases shows that infection of biting insects is not unlikely. The search for an insect vector is limited to biting insects which have a sterile alimentary tract and whose distribution bears some relation to that of the disease, two conditions which in Catania are filled by sandflies. The distribution of *Phlebotomus perniciosus* in Italy coincides very closely with that of Kala Azar. Like the disease, it is absent at high altitudes in Sicily, is rare in the interior and is commonest on the coastal plain. The distribution of *P. papatasi* is much wider than that of the disease, and it is common at altitudes of 1,100 metres where Kala Azar does not

occur. The distribution of a local race of *P. major* in Catania is more restricted than that of the disease, but it is common in two intense foci.

P. perniciosus, *P. major*, *P. papatasi* and *P. sergenti* were fed on a Chinese hamster infected with *Leishmania infantum*. The infection rates were 96·8 per cent. in *P. perniciosus*, 92·3 per cent. in *P. major*, 3·5 per cent. in *P. papatasi* and nil in *P. sergenti*. It is therefore obvious that *Leishmania infantum* is far better adapted to *P. perniciosus* and *P. major* (both sandflies of the *Major* group) than to *P. papatasi* and *P. sergenti*. In the case of *P. papatasi* fed on bone marrow which showed one parasite to 40 fields, a much larger number than that found in the peripheral blood of human cases, the infection rate was nil. In sandflies fed on infected bone marrow showing 7 parasites per field it was never higher than 26 per cent. *P. papatasi* can, therefore, be practically excluded as a vector of Kala Azar in Sicily.

Out of 63 *P. perniciosus* fed on 5 cases of Kala Azar, 4 sandflies were found infected, and a wild *P. perniciosus* caught in a Kala Azar house was found to be naturally infected with *Leishmania*. The infection rate in *P. perniciosus* was one out of 751 in insects caught in or near Kala Azar houses. Out of 3839 *P. papatasi* caught in or near Kala Azar houses none were found infected with *Leishmania*. Out of 130 *P. papatasi* fed on 6 cases of Kala Azar none were found infected.

P. perniciosus is therefore considered to be the important carrier in Sicily because of its distribution, its infection after feeding on human cases and on an infected Chinese hamster, and the extension of the infection into the teeth of the epipharynx.

Fifteen sandflies, *P. perniciosus*, were placed in a Hertig apparatus 5 to 13 days after the infecting feed on a Chinese hamster and in six instances flagellates were recovered from the capillary of the Hertig apparatus. These flagellates could only have entered the capillary from the proboscis and this experiment suggests very strongly that flagellates from *P. perniciosus* can enter a vertebrate host in the absence of any active interference on the part of the latter. This is in accord with the most striking character of Mediterranean Kala Azar, namely, its relative frequency in infants under 12 months who can hardly be expected to crush insects.

The local race of *P. major* is also a possible carrier, but it cannot be the main carrier, for its distribution is not nearly as wide as that of the disease.

A study of the bionomics of *P. perniciosus* showed that this sandfly is present throughout the whole sandfly season, but is commonest from the middle of August to the middle of October and particularly in the second half of this

period. Infections are therefore possible from June to October, but are most likely to occur from the middle of August to the middle of October.

The offspring of the third and fourth generation of *P. perniciosus* hibernate as fourth stage larvæ even if the eggs and larvæ are kept at 30° C., the optimum temperature for the development of *P. perniciosus* of the first two annual generations. A temperature of 30° C. is harmful to the hibernating larvæ and produces a mortality of 70 to 90 per cent. Hibernation is not simply an adaptation to low temperatures, but is an essential part of the life cycle of Sicilian *P. perniciosus*. *P. perniciosus* feeds more readily on mules and cows than on man, and more readily on man than on laboratory animals.

The visceral *Leishmanias*, in so far as they have been studied, appear to be particularly adapted to sandflies of the *Major* group.

The sandflies of the Mediterranean basin are listed, two new species, *P. macedonicus* and *P. canaaniticus* and a new variety *P. major* var. *syriacus* are described.

The distribution of the *Major* group in relation to that of Mediterranean Kala Azar is described and the following species are particularly suggested for further investigation: *P. macedonicus*, a race of *P. chinensis*, *P. ariasi*, *P. vesuvianus*, *P. perniciosus* var. *tobbi* and *P. major* var. *syriacus*. *P. macedonicus* is considered to be the most likely carrier in Macedonia. In the Sudan an unknown species of the *Major* group is probably the carrier of Kala Azar.

A study of *L. infantum* in *Cricetulus griseus*, *Cricetus auratus*, *Microtus guntheri* showed that the pathology is similar to that found in Chinese hamsters infected with *L. donovani*. No cutaneous ulcers developed although the skin was heavily infected, there was no permanent periartritic swelling, parasites were rare in the general circulation and the visceral infection did not disappear spontaneously.

In Catania the gecko *Tarentola mauretanica* was found to be free from *Leishmania* infection. Attempts to infect geckoes by inoculation of *L. infantum* from infected human bone marrow and cultures gave negative results. This gecko can, therefore, be excluded as a reservoir of *L. infantum*.

Phlebotomus minutus and *Leishmania tarentolæ* are both absent in Catania and both present in Tunis and Algeria. The possibility of *P. minutus* being the carrier of *L. tarentolæ* is therefore to be considered.

Seventeen out of 49 specimens of *T. mauretanica* were found to be infected with *Trypanosoma ptyodactyli*, and *P. parroti* and *P. papatasi* were infected with the trypanosome by feeding on geckoes. The flagellates in these sandflies tended to an anterior position. *P. papatasi* can be practically excluded

as a carrier of the trypanosome, for it does not feed on geckoes in nature, as none of the 3,839 wild *P. papatasi* dissected contained nucleated red cells in the stomach.

A female of *P. parroti* was found naturally infected with a trypanosome resembling *T. pyodactyli* in artificially infected sandflies. The infection extended into the pharynx. *P. parroti* feeds on geckoes in nature and it is probably the carrier of *Trypanosoma pyodactyli*.

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*The Reaction of Living Tissues to Homogeneous X-Radiation
Produced by Crystal Diffraction.**

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[PLATE 19.]

Introduction.

The adaptation of the X-ray spectrometer to a biological investigation has been described in previous papers (1925 and 1930). The allantoic membrane of the embryo chick was used as a standard tissue and effects or reactions were obtained consisting of hypertrophy or proliferation and atrophy or destruction, the latter representing a higher degree of change associated with a more intense stimulus.

The energy available after crystal diffraction is so small that it is necessary to make certain assumptions when biological changes can be obtained in a reasonable time of exposure. It would appear that there is a neutralising action between frequencies and that the action of any one frequency is selective with respect to another. There is ample experimental evidence of the neutralising action which was termed antagonism and of a selective action (1930, *a* and *b*).

The present paper is concerned with an attempt to extend the above phenomena to other tissues. In accordance with the above supposition it is only necessary in a preliminary investigation to obtain definite X-ray effects and the main problem involved is the proof that abnormalities observed are due to X-radiation.

I.—Experiments on Mammalian Skin.

Technical Procedure.—The skin of the white mouse was used as a typical mammalian tissue. The X-ray spectrometer only furnishes a small pencil of rays and complete immobilisation was necessary, for with any movement no one area would receive an effective dose.

* This work was carried out under the control of the Cancer Research Committee of the University of Sydney and with the aid of the Cancer Research and Treatment Fund.

Two stitches were passed through the skin so that a small fold would be held immobile in a wooden frame and a small drop of carbol fuchsin was placed on the hair to indicate the irradiated region. After an interval of 7 days, about 1 sq. cm. of skin was removed and sectioned serially for microscopic examination. To avoid any fallacy the hair was not disturbed until after fixation, and in this condition no definite change could be observed by the naked eye.

The above procedure was adopted for four control preparations and, as indicated by the letter O in Table I, no abnormality was observed. Thus acute flexion of the skin for a short period of time did not produce any visible reaction. Unfortunately, in some of the irradiated specimens a reaction due to the insertion of a stitch was observed in the area sectioned.

Three additional controls were therefore made for the investigation of stitch reactions, which are designated by the letter S in Table I.

Experimental Details.—The first 22 experiments were performed with the low-powered apparatus used for the exposure of the allantoic membrane. No conclusive result was obtained and the following description applies to the later work, series A, Table I. A water-cooled Coolidge deep therapy tube was used, energised by a Gaiffe Gallot constant potential generator. The focal spot was found to be annular and the tube was rotated until a line approximately 2 mm. broad was presented to a "lathe bed" spectrometer. The arrangement of the apparatus is shown in fig. 1, slits A and A' being 2 mm. wide and slit B,

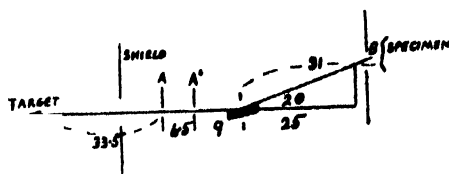


FIG. 1.

3 mm. wide. A calcite crystal 2 mm. thick was used and the angle of reflection was estimated as a tangent. The divergence of the beam was approximately 1/150 giving a greater degree of homogeneity than the 1/50 divergence used for the experiments with the allantoic membrane. The specimen was exposed for 3 hours at 10 m.a. and a potential of 55 kv., some of the work being done at 15 m.a. and 80 kv. Various experiments were made at different wave-lengths, according to the details given in Table I.

Table I.

Approximate wave-length.	Remarks.	Total experiments.	O.	S.	?	H.
	Control	4	4	—	—	—
	Stitch reaction	3	—	3	—	—

SERIES A.

0.32		6	—	1	4	1
0.42		2	2	—	—	—
0.53		6	8	1	1	1
0.68		4	—	—	3	1
0.79		13	1	5	2	5
0.84		4	—	1	1	2
Total Series A		35	6	8	11	10

SERIES B.

0.32	Stitch excluded		1	—	1	1
0.42			1	—	—	1
0.53			2	—	1	—
0.79			—	—	—	2
Total Series B		10	4	—	2	4

SERIES C.

0.5	Series C.	8
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"O," no reaction. "S," stitch reaction. "?," doubtful. "H," hypertrophic reaction.

Nature of Abnormalities.—Certain abnormalities were observed in the irradiated specimens and a typical example is shown in fig. 2 (Plate 19). There is a region in which the epithelium is considerably thickened in comparison with the normal, which is also included in the figure. The epithelial cells are also enlarged and contain prominent nuclei as evidence of their increased activity, but hair follicles are comparatively rare and somewhat abnormal in this region. (A fallacy arising from an oblique section can be excluded by tracing an abnormal region through a series of sections.)

Changes are also visible in the deeper tissues corresponding to the thickened epithelium. There is a considerable fibrosis and an infiltration by round cells and spindle-shaped cells, whilst engorged capillaries are frequently observed.

The above-mentioned abnormality is represented by the letter H, while in some cases the result was represented by ? because in a critical examination

one did not feel justified in definitely attributing changes to X-radiation. Some specimens were excluded because they contained a reaction which could definitely be associated with the insertion of a stitch (S). The latter closely resemble a supposed X-ray reaction and no criteria have been found for distinguishing with certainty between them. The stitch reaction, however, occurs at the extreme end of the section and two reactions may appear when an unduly large piece of tissue has been removed. Such reactions are, in general, clearly circumscribed and they usually show a characteristic break in the subcutaneous muscle bundles.

The X-ray reaction is more diffuse and the deep infiltration less marked. It is also situated roughly in the centre of the portion of the skin removed for examination. In the next series (B) the technique was modified by marking the position of each stitch with a dye so that these regions could be avoided with certainty in removing the specimen for examination. Although the main source of confusion was avoided, some very slight reactions were designated by a query.

It appeared desirable to employ an even larger dose, so a multiple spectrometer was constructed to give a fixed wave-length of $0.5 \text{ \AA}$. according to the details in fig. 3. A fine focus Universal Coolidge tube was used and the apparatus was placed so that the focal spot formed a circular source of radiation

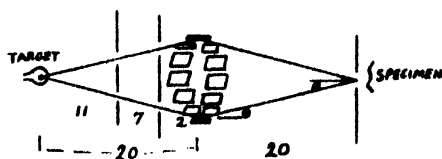


FIG. 3.

about 2 mm. in diameter. Two annular slits were arranged so that a cone of rays should impinge on a series of calcite crystals placed round a circular form. The object to be irradiated was then placed at the image of the source. Practical difficulties of construction limited the output of homogeneous radiation attained and for the same reason a considerable frequency range was used owing to the comparatively large aperture of the annular slits. Nevertheless, an area of about 1 sq. cm. was covered by a radiation several times the intensity previously used. Eight experiments were carried out with the apparatus (Series C, Table I), giving an exposure of 2 hours at 3 m.a., and an interval of 14 days was allowed before removing the skin, under the impression that reaction would be more fully developed. Some of the sections were very poor and four experiments could only be designated with a query.

Relationship to Experiments with Allantoic Membrane.

An X-ray reaction was inferred in about 30 per cent. of series A, 40 per cent. of series B and 25 per cent. of series C. It is not possible to say that these figures represent a proportion of radiosensitive animals because the technical difficulties of identifying a particular area on the very elastic skin are great and, as mentioned above, all doubtful cases were excluded.

It is to be remarked that the reactions, if due to X-radiation, represent a response to a dose just above the threshold and in accordance with previous experience (1930, *b*) one might expect some specimens to show no reaction owing to variation in sensitivity. It may also be mentioned that the allantoic membrane which has received a dose just above the threshold shows a circular region of hypertrophy which is much smaller than the irradiated area (1930, *c*). The same condition appears to apply to the mouse skin and the method of drawing up a fold during exposure might well lead to confusing appearances. The positive results in series C showed the largest area and greatest degree of change, and it appears justifiable to assume that a reaction has been produced in the mouse skin by the method of crystal diffraction. This reaction involves a proliferation of the epithelium to which the term hypertrophy may be applied so that homogeneous radiation must first cause a stimulation in a similar manner to the changes in the allantoic membrane. This is in contrast to the action of mixed radiation, which does not usually lead to stimulation.

In a previous paper on the time factor it was deduced that the mouse skin might react in the same manner as the allantoic membrane, but the resistance would be much greater owing to a more effective repair (1930, *d*).

The present results are in accord with the above supposition. No definite results could be obtained with the low-power apparatus used for the allantoic membrane (1930, *a* and *b*), and the water-cooled tube and spectrometer described here gave a hypertrophic reaction in the allantoic membrane in $\frac{1}{2}$ hour at 6 m.a. (1930, *b*), whilst the mouse skin required a 3-hour exposure at 15 m.a. or about 15 times the dose. The erythema dose for mouse skin, using direct unfiltered radiation through the same system of slits, was also represented by an exposure of approximately 3 hours at 15 m.a. and as the relative intensities are about 1 : 1000, this ratio represents the potency of the two radiations. As a similar ratio was obtained for the allantoic membrane, it is also about 15 times more sensitive to mixed radiation than the mouse skin. It must be remembered that the direct rays contain all the various homogeneous components in much greater intensity than those obtained after crystal diffraction.

There is thus evidence that the mouse skin shows antagonism and selective action as a dual phenomenon, although the results in Table I are not sufficient to demonstrate any selective action.

II.—*Experiments with Representatives of the Lower Phyla.*

A prolonged and rather unsatisfactory investigation has been made on the action of homogeneous X-radiation on representatives of the lower phyla. Eight experiments were made with sea anemones (*Paractis papaver*, Drayton) and 31 with narcotised tadpoles placed in a drop of water, but no conclusive result was obtained. Finally, curarised frogs were placed so that the X-rays fell on the skin of one leg, and sections were made after 7 and 14 days' interval. The irradiated patch of skin was usually adherent to the subjacent muscle bundle and microscopic examination showed a region in which the epithelium was considerably thickened and frequently thrown into papilliform processes, whilst there was a corresponding infiltration of the deeper tissues with small round cells (fig. 4, Plate 19).

Adopting the previous classification, four experiments with the water-cooled tube (fig. 1) gave the following result, H, H, ?, O, whilst 10 experiments with the ring of crystals showed six H, two ? and two O, and one control specimen was also sectioned. Thus, 57 per cent. showed a definite abnormality which may be described as hypertrophy and, in accordance with the previous discussion, it may be assumed that this change corresponds to the reactions in the allantoic membrane and mouse skin.

III.—*Experiments with Chick Tissues grown in vitro.*

A considerable amount of work with plasma cultures was carried out in collaboration with Dr. J. Byrne, without any conclusive result. Finally, chick heart cells were grown in amniotic fluid, which serves as a physiologically neutral fluid, using a method described in a previous paper (1927). An excellent and very reliable growth is obtained in 24 hours, but the cells degenerate completely in the next 24 hours and an attempt was made to show that a 2 hours' exposure with the lathe spectrometer, water-cooled tube, etc., hastened that process. In place of the glass cavity slide, brass plates were used with a circular aperture closed by two thin coverslips and the beam of rays did not impinge on the metal, so that there would be very little scattering with consequent loss of homogeneity. This is likely to be very important, because the presence of scattered radiation prevented a reaction in the allantoic membrane (1930, a), and ideally one would avoid even a glass coverslip.

The use of a fluid medium makes a horizontal position of the slide desirable, but it is very inconvenient to place an X-ray spectrometer in a vertical position and a compromise was effected by placing the culture at an angle of 45°. A control preparation was also placed in a similar position about 1 cm. away so that it would be screened from the rays.

The preparations were fixed and stained 4 hours after irradiation, and conclusions were made only if both cultures were satisfactory and showed the same degree of growth. From the 10 pairs examined, the controls showed a normal growth with extended amoeboid cells and large nuclei with one or two distinct chromatin aggregations (Table II). A few of the cells, particularly at the

Table II.

A. Controls	N N — — — N — — S N N — — S N N N
B. Homogeneous radiation 0.5 Å.	D D — — — D D D D D D D D D D D
C. Mixed radiation approxi- mately 200 times dose of B	N N N N S — — — N N discontinued

N, normal. D, degeneration. S, slight degenerative change. —, indeterminate.

periphery, showed a commencing degeneration. The most characteristic change after irradiation was a shrinkage of the nuclei and the more advanced degeneration and fragmentation of the cytoplasm could be appreciated at a glance (fig. 5, Plate 19). A series of 7 cultures were exposed to mixed radiation, giving approximately 200 times the dose (by a rough photographic comparison) and these specimens showed no obvious difference from the control preparations. It appears, therefore, that the dual phenomenon of selective action and antagonism have been demonstrated in the case of *in vitro* cultures.

Conclusions.

It is perhaps fortunate that the allantoic membrane was selected at an early period (1925) for the investigation of the action of homogeneous X-radiation, because all other forms of tissue have so far been associated with much greater experimental difficulties. The ultimate extension of the early results to other tissues was inferred from the fundamental nature of X-ray action which is associated with the atom and certain other features in connection with the "time factor" (1930, d) and the area of tissue irradiated (1930, c) have given further weight to this hypothesis. The histological appearances observed in some of

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the present experiments furnish the first direct evidence that the dual phenomenon of antagonism and selective action in the allantoic membrane applies to mammalian tissues, to amphibian tissues, and to a culture grown *in vitro*. A later paper will contain the results of a preliminary investigation with tumour tissues, and even now there can be little doubt of the ultimate general application of the phenomena under discussion. An interesting point is the existence of a stimulation dose for homogeneous radiation, the first change in the frog and mouse as well as in the allantoic membrane being a hypertrophy.

As previously discussed, the mouse skin is about 15 times more resistant than the allantoic membrane to both mixed and homogeneous radiation and the frog skin appears to have a similar high resistance.

It would not be possible to compare the resistance with that of the short-lived cells in the cultures used, but the variation in latent periods, before any visible change takes place, offers an interesting comparison. In the highly organised tissues of the mouse, presumably with a high rate of repair, several days were necessary to demonstrate the obvious tissue changes termed the biological stage of an X-ray action. The allantoic membrane, which is a temporary embryonic structure, shows definite tissue changes in 1 or 2 days (1930, *a*), and the heart cells growing artificially *in vitro* showed a change in a few hours.

I wish to express my thanks to Professor C. W. Stump in connection with 148 specimens which were sectioned in the Department of Histology.

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- (1930, *d*). 'Proc. Roy. Soc.,' B, vol. 107, p. 308.

EXPLANATION OF PLATE 19.

- FIG. 2.—Irradiated Mouse Skin (normal on right).
- FIG. 4.—Irradiated Frog Skin (normal on right).
- FIG. 5.—Irradiated Culture (control on right; note mitosis).

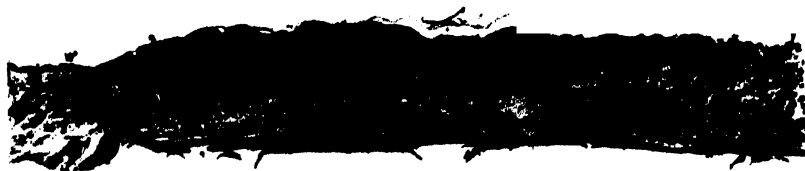


FIG. 2. Irradiated mouse skin (normal on right).

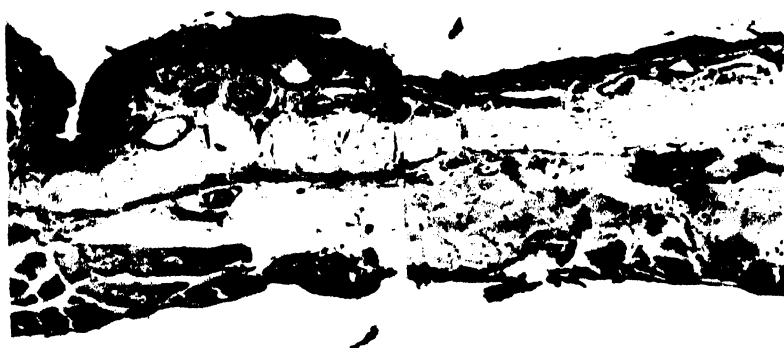


FIG. 4. Irradiated frog skin (normal on right).

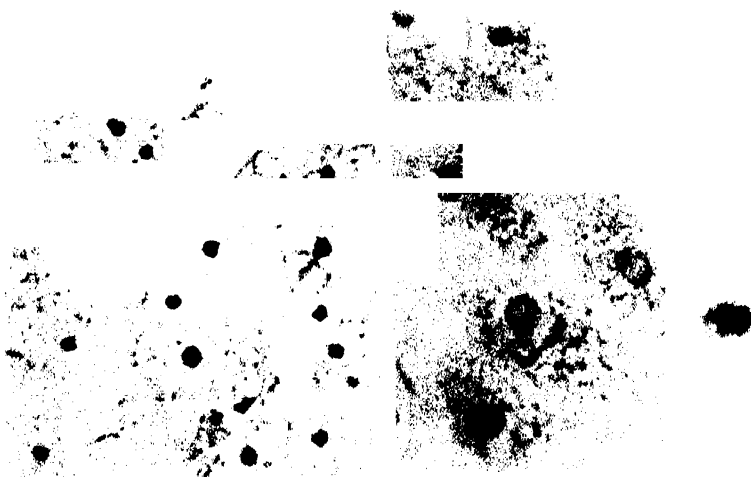


FIG. 5.—Irradiated culture (control on right, note mitosis).

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*The Potential Difference Across the Surface Bounding the
Unfertilised Egg of the Brown Trout.*

By R. J. PUMPHREY, B.A., Zoological Laboratory, Cambridge.

(Communicated by J. Gray, F.R.S.—Received May 2, 1931.)

Most living cells maintain in their interior a concentration of electrolytes different from and, in general, higher than that of the surrounding medium. Most living tissues when excited or injured develop a difference of electrical potential between the excited or injured part of the surface and the remainder. It has long been supposed that these phenomena are related; and the injury and action potentials have generally been explained by assuming a difference of concentration between the interior and the exterior of the cell and assigning arbitrary properties to the cell surface to account for the sign and magnitude of the potentials found.

Recently the work of Michaelis (1927-28) and others has provided inanimate membranes which have some of the properties assigned to the cell surface, and a detailed comparison is obviously desirable. Measurements of the potential developed by single cells have been extremely few and in the majority of cases qualitative rather than quantitative. It was therefore considered of interest to examine in detail the bioelectrical properties of a single cell. In this paper the effects of changes of the composition and concentration of electrolytes in the surrounding medium on the potential across the surface of the unfertilised trout's egg are examined, and it is concluded that there is a rather close resemblance between the behaviour of this surface and that of the negatively charged collodion membrane of Michaelis.

Material.—The unfertilised trout's egg was chosen for these experiments because it was large enough to handle easily, fairly hardy and easily obtainable. Moreover, certain facts were already known about its behaviour (Gray, 1919-21). It consists of a sphere of yolk surrounded by a layer of protoplasm which is very thin except for a polar cap which forms the blastodisc. This layer in turn is surrounded by a thin but tough and elastic membrane, the chorion. The whole egg is about 4 mm. in diameter. The internal concentration of electrolytes is equivalent to 0.12 M. sodium chloride. The only salts present in significant amount in the normal environment are sulphates and carbonates

of calcium and magnesium equivalent to 0.002 M. Ca^{++} . This large difference in concentration is maintained so long as the egg remains alive.* If the egg dies, or is killed, salts rapidly diffuse out from it and a white precipitate of globulins appears inside it. In what follows, eggs which remained clear and translucent during the period of experiment are termed alive. Eggs which turned partially or wholly white and opaque are termed dead.

Method.—The method of measuring the potential was a modification of that used by Osterhout (1928) for the large unicellular alga, *Valonia*. The tip of a micro-pipette, filled with M/10 KCl, was inserted into the egg in such a way as enabled an effective seal to form round the point of puncture. Under such conditions the whole of the egg-surface retained its normal degree of impermeability to electrolytes. In those cases in which the pipette caused irreversible injury the latter could readily be detected by the precipitation of intracellular globulin which invariably follows the diffusion of salts through an injured surface.

The difference of potential was neutralised by a slide-wire potentiometer using a Lindemann electrometer as a null-point instrument. An egg was supported in a glass vessel containing tap water and the micro-pipette con-

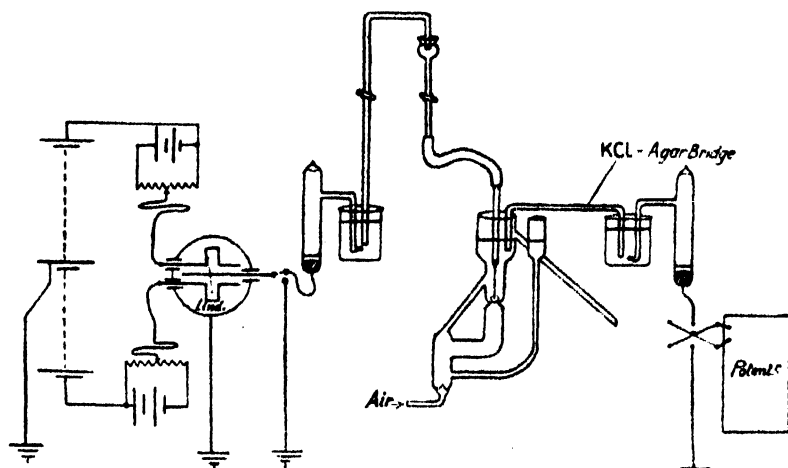


FIG. 1.

taining M/10 KCl inserted into it. The micro-pipette was connected to the needle of the electrometer and the tap water to the potentiometer by a pair of saturated calomel electrodes. The other terminal of the potentiometer

* Analysis of eggs kept for some time in tap water suggest that there may be some exchange of ions between the egg and its environment.

was connected to a properly earthed sheet of metal on which all the apparatus was mounted. The electrometer was used with a periscope and could be made to give a sensible deflection for 0.5 mv. The system gave zero potential ± 1 mv. in the absence of the egg when the pipette dipped directly into tap water and the contained KCl flowed out under slight pressure. Substitution of other solutions for the tap water had no measurable effect on the potential when the KCl-agar bridge was fresh except in the case of strong solutions of calcium and magnesium chlorides. In M/5 calcium or magnesium chloride the bridge became negative to the solution by 3 to 5 mv. This has been allowed for in tabulating the results. A fresh bridge was used for each run.

The vessel used was designed to give effective stirring and to facilitate changing the external solution with the minimum mechanical agitation of the egg. It proved very satisfactory in both these respects. If the egg survived puncture it remained alive for many hours and sometimes for days in tap water. In the best conditions as many as half the punctures were successful.

Sumwalt (1929) measured the concentration potential across the chorion of *Fundulus* eggs and found that this membrane behaved as if almost impermeable to anions. This is clearly not the case for the trout's egg, since the chorion does not hinder the free diffusion of salts from an egg which has been killed. Attempts to remove the chorion from the egg without injury to the latter were unsuccessful. It was, however, found possible to remove pieces of the chorion intact and to mount them so that different solutions could be applied to the two sides. When the chorion separated solutions of M/10 and M/100 KCl, the potential difference between the two solutions, did not differ within the limits of error from the free diffusion potential expected. It was therefore concluded that the effect of the chorion could be neglected.

Results.—The potential difference across the egg surface immediately after puncture was generally small and if the egg died remained small, about 5 to 7 mv. (In all cases, unless explicitly stated otherwise, the inside of the egg is negative to the surrounding solution.) If the egg lived, the P.D. changed and after 10 minutes or so became constant generally at 13 to 18 mv. Rarely values as high as 40 mv. and as low as 2 mv. have been recorded. If the tap water in the vessel was renewed at intervals and kept stirred, this constant potential was maintained generally for several hours. The difference in the steady value observed in different eggs illustrates clearly that the potential difference across the cell-surface is a function of the state of the surface itself as well as of the ionic difference in concentration between the interior of the cell and the external fluid. For a given difference of concentration on the two sides it

may be possible to form an estimate of the maximum potential difference which can be set up across a given cell surface, but it is impossible to foretell the value of the potential which actually exists.

If, after a steady potential had been reached in tap water, distilled water was substituted as an external medium, the potential difference rose sharply to 60-100 mv. and then fell off roughly exponentially with time (fig. 2A).

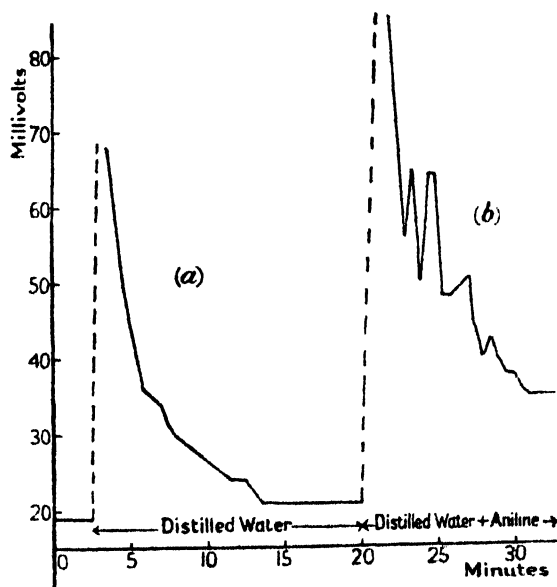


FIG. 2.

The fall was at first attributed to salts diffusing from the egg sufficiently fast to decrease the concentration gradient at the surface. Conductivity measurements performed by Mr. Gray, however, do not indicate any measurable diffusion from the egg in distilled water. The fall therefore remains unaccounted for. The potential can be maintained at a high level apparently indefinitely by repeated washing with distilled water. It is certain that exposure to distilled water makes the eggs very much more delicate and sensitive to handling. A solution of calcium chloride isotonic with tap water does not have this effect. It is not yet known whether other ions can replace calcium in this respect.

If a healthy egg is exposed to a saturated solution of aniline in distilled or tap water the egg rapidly dies, salts diffuse outwards, and the egg becomes opaque. The effect of these processes on the potential difference across the egg membrane is illustrated by the following experiments. An egg, after

puncture with the electrode, gave a steady value of 20 mv. when tap water was the external medium; the latter was then changed to distilled water, whereupon the potential difference rose sharply to 68 mv. and then fell away to its original value of 20 mv. The external solution was then changed to a saturated solution of aniline in distilled water; again the potential difference rapidly rose to a high value (see fig. 2B) and began to decrease again. In this case, however, the decline of the potential was marked by very distinct fluctuations indicating that rapid and extensive changes were occurring in the permeable properties of the membrane, which changes do not occur as long as the egg remains healthy.

The potential difference across the membrane of a dead egg was also measured as follows. An egg which had survived puncture was killed by a saturated solution of aniline in tap water and the whole of the diffusible salts in the egg were allowed to leave the egg until further washing with fresh tap water did not alter the potential. The potential then measured was found to be constant at a value between 20 and 24 mv.* This residual potential of the dead egg may be due to the presence of large ions in the egg which are unable to diffuse through the the surface of the egg or through the chorion. In this case, according to the Donnan equation, it should tend to zero as the concentration of diffusible ions is increased. This did not seem to be the case, but possibly insufficient time was allowed for the system to reach equilibrium.

The Potential Difference in varying Concentrations of different Salts.

The properties of the intact cell surface can, to some extent, be investigated by observing the potential difference set up by the system when the external solution consists of salts in known concentrations.

I. *Uni-univalent Salts.*—Solutions of sodium and potassium chlorides were made up in the following concentrations: M/3125, M/625, M/125, M/25, M/5, and applied to the live egg in that order. Solutions more dilute than M/3125 were tried, but the potential became increasingly unsteady with increasing dilution and showed a tendency to fall off with time as in distilled water. The value shown for the potential in the most dilute solution is the highest obtained after repeated washing. In solutions stronger than M/3125 the P.D. rapidly reached a steady value unaltered by further washing. The results are shown in Table I.

* The low values for the P.D. in tap water when the egg died as the result of manipulation (see p. 513) are attributed to an imperfect seal round the point of entry of the pipette.

Table I.

Concentration of external solution.	Sodium chloride.			Potassium chloride.		
	Potential difference observed.		Average P.D. for $c_1/c_2=5$.	Potential difference observed.		Average P.D. for $c_1/c_2=5$.
	Egg 1.	Egg 2.		Egg 3.	Egg 4.	
M/3125	-32	-30	10.5	-55	-63	23.0
M/625	-20	-21		-34	-38	
M/125	-9	-11	10.5	-18	-17	18.5
M/25	-2	-2	8.0	-8	-8	9.5
M/5	0	0	2.0	-4	-4	4.0

The sign is that of the interior of the egg with reference to external solution.

The figures in the first two columns are actual readings to the nearest millivolt ; the sign is that of the interior of the egg. The figures in the third column for each salt are the average changes of potential for each increase in concentration. For the sake of clarity the results of only two runs are shown for each salt. In most cases more experiments were done with nearly identical results. In one or two cases the change of potential was substantially less than the average, and approximated to the values found for the dead egg although the egg appeared to be healthy. These results have been discarded.

It was found possible to obtain substantially the same results from a second run with the same egg, but in the second run steady readings were only obtained after more prolonged washing. This is probably due to the fact that yolk tends to be sucked up into the pipette and the egg gradually shrinks away from the chorion, leaving a space containing solution which may take some time to come into equilibrium with the external solution.

Similar experiments were made with eggs which had been killed with aniline subsequent to puncture and then allowed to reach a constant potential in tap water. The change of potential with increased concentration was considerably less than for the live eggs (fig. 3). The readings were taken as rapidly as possible and it is probably safe to assume that no appreciable change in the internal concentration of the egg took place during the run.

The results indicate that the surface which is responsible for the observed potential differences behaves as though it were more readily traversed by

cations than by anions, and this conclusion is supported by the results of similar experiments carried out with sodium and potassium sulphates instead of

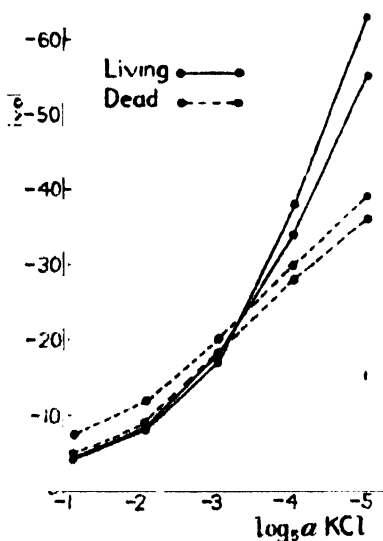


FIG. 3.

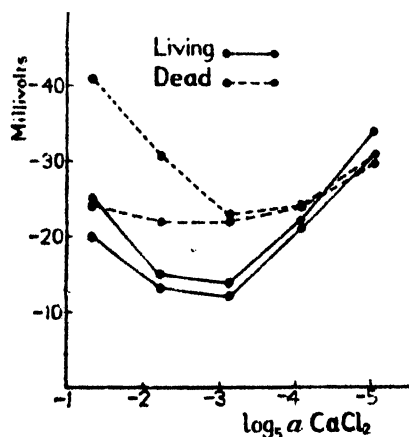


FIG. 5.

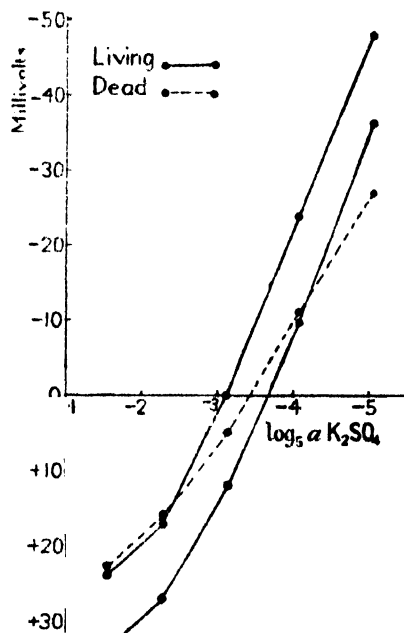


FIG. 4.

In these three graphs the potential difference is plotted against the logarithm of the activity. The activity factors were obtained by interpolation from freezing point data (Glasstone).

chlorides. It will be noted (Table II and fig. 4) that the change of potential caused by a fivefold change in molecular concentration is higher than that produced by the corresponding chlorides.

Table II.

Concentration of external solution.	Sodium sulphate.			Potassium sulphate.		
	Potential difference observed.		Average P.D. for $c_1/c_2=5$.	Potential difference observed.		Average P.D. for $c_1/c_2=5$.
	Egg 5.	Egg 6.		Egg 7.	Egg 8.	
M/3125	-30	-36	18.0	-36	-48	25.0
M/625	-14	-16		-10	-24	
M/125	+4	+2	18.0	+12	0	23.0
M/25	+13	+15	11.0	+27	+17	16.0
M/5	+14	+17	1.5	+34	+24	7.0

The sign of the P.D. is that of the interior of the egg with reference to the external solution.

In contrast with the effects of salts with uni-valent cations are those obtained with the chlorides of calcium and magnesium. In these cases the minimum difference in potential is reached at concentration of about M/125, and any further increase in concentration either does not alter the potential or renders the interior of the egg more negative. This is true both for the living and the dead egg.

Table III.

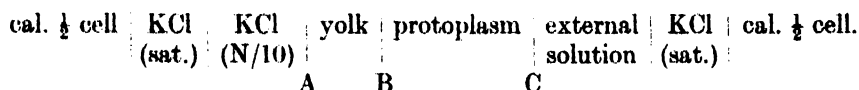
Concentration of external solution.	Calcium chloride.			Magnesium chloride.		
	Potential difference observed.		Average P.D. for $c_1/c_2=5$.	Potential difference observed.		Average P.D. for $c_1/c_2=5$.
	Egg 9.	Egg 10.		Egg 11.	Egg 12.	
M/3125	-31	-34	10.0	-38	-41	11.5
M/625	-21	-22		-27	-29	
M/125	-12	-13	9.0	-17	-18	10.5
M/25	-12	-13	0.0	-18	-20	-1.5
M/5	-19	-18	-7.0	-21	-25	-4.0

The sign of the P.D. is that of the interior of the egg with reference to the external solution.

In view of the results obtained by Fujita (1925) with protein membranes and by Amberson and Klein (1927-28) with the dead frog skin, it is important to note that the potential difference across the egg surface is peculiarly insensitive to variations in the hydrogen-ion concentration of the external solution. Solutions of M/10 KCl were buffered to p_H 7.4, 6.0 and 4.5 with N/100 mixture of KH_2PO_4 and K_2HPO_4 and applied successively to the external surface of the same living egg. The observed potential was the same in each case within 2 mv.

Discussion.

In attempting to define the properties of the egg-surface it should be remembered that no significance can be attached to the absolute values for the potentials measured since the system is asymmetrical, consisting of a chain which can be represented diagrammatically as follows:—



There may be potentials at the junctions A, B and C and the P.D. which is actually measured is the sum of these. It is impossible to estimate the sign and magnitude of any one individually. From the general consistency of the results, however, it seems probable that the potentials at A and B once established remain constant during the experiment and that changes in the potential of the chain when the external solution is changed depend solely on changed conditions at C.

During recent years the effects of certain inanimate membranes in modifying the diffusion potential between two different solutions of electrolytes have been studied by various authors. These effects have been shown to depend partly on the size of the pores and partly on the capacity of the material composing the membrane to combine with or adsorb preferentially ions of one sign, thereby acquiring a charge. The effect of this charge must be to repel ions of the same sign and it is supposed that when the size of the pores approaches ionic dimensions the repulsion is sufficient completely to prevent their penetration. Practically it has been shown that for dilute solutions of uni-univalent electrolytes the diffusion potential across the finest pored collodion membranes is such as would be expected theoretically if anions were unable to penetrate.

From an inspection of the table for uni-univalent electrolytes above, it is obvious that the surface of the trout's egg resembles in its behaviour a collodion

membrane which approaches but does not reach the limiting condition of complete impermeability for anions. In view of the fact that, under normal conditions, the rate of exosmosis of salts from a living egg is extremely slow, it follows that although cations traverse the membrane with greater ease than do anions, yet the absolute velocity of both must be very low.

The surface of the trout's egg resembles the collodion membrane and differs from the protein membranes which have hitherto been described in that the concentration potential across it is not appreciably affected by change of p_H (provided the concentration of hydrogen ions is small compared with that of other cations). It is therefore probable that the surface layer does not consist of proteins as has been supposed by some authors. Amberson and Klein (*loc. cit.*) found that for frog skin killed by exposure to acid solution changes in p_H had a very marked effect both on the concentration potential and the rate of electro-osmosis across the skin, the sign of the former and the direction of the latter being reversed at about p_H 5. It seems likely, however, that in the dead skin, at any rate, the electrical properties are determined by the intercellular matrix.

Theoretically, it would be expected that if the condition of the membrane remained unchanged, the change of potential for a fivefold increase in activity should be constant over the whole range of concentration, *i.e.*, the potential difference should be proportional to the logarithm of the activity of the external solution. It is evident that this is not the case. As the concentration is increased $dE/d \ln a$ decreases. This is true for all the salts but is most evident for those containing bivalent cations. The same phenomenon was observed by Michaelis for uni-univalent electrolytes with the collodion membrane. It seems reasonable to suppose that it is due to a reduction of the total negative charge on the surface layer by the increasing absorption of cations as the concentration is increased. If the concentration of cations in the external medium is sufficiently large, the membrane may carry a positive charge and in this condition the cations in the environment will be repelled whilst the anions can traverse the membrane, thereby rendering the lower concentrations electronegative. This discharge of the membrane is precisely analogous to the discharge of a gold sol, for example, by cations, and the effects of bivalent cations are evident in both cases at much lower concentrations than the effects of univalent cations. This explanation has not yet been put forward for the decreased potential across the collodion membrane in strong solutions. Michaelis' measurements (Weech and Michaelis) of the diffusion potential across the collodion membrane in calcium chloride solutions were made for

one pair of concentrations only and he attributes the low potential found to the large size of the hydrated Ca^{++} ion. Measurements at other concentrations are necessary before this can be accepted as final.

The smaller change of potential with increased concentration when the egg is dead is possibly to be attributed to an increase in the size of the pores since diffusion across the surface is relatively very rapid after death.

Summary.

(I) The e.m.f. across the surface of the trout's egg is 13-18 mv. when the external medium is tap water and from 60-100 mv. when it is distilled water. In both cases the interior of the egg is electronegative.

(II) When the cell is dying the e.m.f. fluctuates. This fluctuation is most apparent in distilled water.

(III) When the external medium is a dilute solution of a non-toxic salt the e.m.f. is a linear function of the logarithm of the activity. The gradient is characteristic of the salt used and its sign is that which would be expected if the surface was more permeable to cations than to anions.

(IV) The e.m.f. is independent of the p_{H} between 7.4 and 4.5.

(V) There appears to be a similarity between the surface of the egg and the molecular seive membrane of Michaelis. An explanation is suggested for the anomalous effect of salts in high concentration on the e.m.f. observed.

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The Heat-tension Ratio in Prolonged Tetanic Contractions.

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It was found by Hartree and Hill (1921) that in tetanic contractions of various durations the relation between heat liberated and duration of stimulus soon becomes linear; from the slope of the curve relating the two variables the absolute values of the heat production were calculated, per centimetre length of muscle, per gramme weight of tension maintained, per second of stimulus, in contractions at various temperatures. No observations, however, were made with tetani longer than 2 seconds.

The isometric time coefficients of lactic acid formation and of phosphagen breakdown (subsequently denoted, following Meyerhof, by the symbols $K_{z(L)}$ and $K_{z(P)}$ respectively) have been determined by various workers and summarised by Meyerhof (1930, pp. 102, 234, etc.). Recently, however, the existence of delayed lactic acid formation following a tetanus, long maintained by Embden and his school, has been confirmed by Meyerhof and his collaborators (1931), who failed to do so in several previous investigations. Lunds-gaard (1931) also has found that the anaerobic delayed lactic acid formation following a 5-second tetanus may be over one-half of the total lactic acid set free. When there is a delayed lactic acid formation amounting to as much as, or more than, 100 per cent. of that occurring during the contraction proper, it is obvious that the values of $K_{z(L)}$ and $K_{z(P)}$ lack significance unless the time from the end of the stimulus at which the chemical determinations are made is specified. The older chemical investigations mentioned thus require revision, which has been given in a recent paper by Meyerhof and Schulz (1931).

In myothermic studies the measurement of heat by the method of maximum deflection minimises the complication of delayed heat production, which latter, moreover, is much smaller than would correspond to the delayed lactic acid formation, since in all probability it represents the surplus energy of lactic acid production unused for phosphagen resynthesis. Now that more precise

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chemical data are available, it seemed desirable to extend Hartree and Hill's work to longer tetani and to see how far we can carry the correlation between thermal and chemical measurements. The present study covers durations of tetanus from 2 to 20 seconds at 20° C., while at 0° C. observations have been made on tetani as long as 10 minutes.

I.—THE NORMAL TENSION HEAT RATIO IN LONG TETANI AT DIFFERENT TEMPERATURES.

The experiments were made mostly on the sartorii of *R. esc.* and *R. temp.* For comparison, other species of frogs and semi-membranosus muscles were used in a few experiments. At 20° C. nearly all the observations were made in nitrogen, at lower temperatures mostly in oxygen. The liberation of recovery heat being very slow, especially at low temperatures, the presence of oxygen does not appreciably affect the measurement of the initial heat. Stimulation was direct and of a frequency low enough to avoid the failure of excitation which occurs in a prolonged tetanus with too high a frequency. At 20° C. a Neon lamp stimulator (see Briscoe and Leyshon, 1929) was used to give a series of condenser discharges at a frequency just sufficient to cause complete fusion of individual responses. At lower temperatures a lower rate of stimulation was used and a rotating contact breaker of the type described by Gerard, Hill and Zotterman (1927) was employed. The heat was measured by the maximum deflection method with thermopiles of the type described by Hill (1928). Tension was recorded simultaneously on a smoked drum by a torsion lever of the usual type.

There is no difficulty in measuring the large heat production, even of a tetanus lasting as long as 20 seconds, by the maximum deflection method; the thermopile-galvanometer system must only be made sufficiently insensitive and slow. In the present experiments a Kipp moving coil galvanometer (Z_c) was used, shunted by a low resistance*; for tetani longer than 5 seconds the surface of the thermopile was covered by a very thick layer of paraffin wax. To reduce the sensitivity still further in experiments at 20° C. a suitable resistance was introduced into the circuit. The proportionality of deflection to heat production may be tested on the dead muscle by electrical heating; a linear relation between the duration of a constant heating current and the maximum deflection obtained should exist up to a time of heating as long as the tetanus to be investigated.

* To reduce its sensitivity and, by overdamping, to make it slower.

The results at 20° C. are summarised in Table I. In the expression Tl/H , T is the mean tension in grammes weight developed and maintained; t is the period of stimulation in seconds; l is the length in centimetres of that portion of the muscle which is calibrated for the heat measurement, and H is the heat liberated in gramme centimetres. The ratio has the dimension of time, and although it has no exact physical meaning it represents in a general way the time scale of the muscle under the conditions of the experiment. Slower muscles give a greater value of the ratio and maintain a contraction more economically. The expression is employed chiefly for convenience in that it affords a ready comparison with the chemical findings of Meyerhof's laboratory. It may be called the isometric time coefficient of heat and represented by the symbol $K_{z(H)}$.

Table I.—The Isometric Time Coefficient for Heat ($K_{z(H)} = Tl/H$) of Normal Muscles at 20.3° C.

Species of frog.	Muscle.	Duration of stimulus.	Number of experiments.	Average Tl/H .
		seconds		
Small Dutch <i>R. esc.</i>	Sartorius	10	2	1.00
"	"	20	2	1.00
"	"	5	4	0.89
Large Hungarian <i>R. esc.</i>	"	5	2	1.19
Small Dutch <i>R. esc.</i>	"	2	3	0.78
<i>Xenopus laevis</i> (S. Africa)	"	2	2	0.97
"	Semi-membranosus	2	1	0.91
Small Dutch <i>R. esc.</i>	"	2	2	0.70
English <i>R. temp.</i>	"	2	2	0.72
Large Hungarian <i>R. esc.</i>	Sartorius	2	2	0.86
English <i>R. temp.</i>	"	2	8	1.00

Comparing the 2-second tetani for the several species of frogs and the two types of muscle, it appears that the sartorius of *R. temp.* is somewhat more economical in maintaining a tension than the sartorius of *R. esc.*, but on the whole the agreement throughout the series is astonishingly close. The higher coefficient for *R. temp.* is perhaps to be related to the observation recorded in the next section, that its sartorius is better able to give long tetanic contractions than that of *R. esc.* The $K_{z(H)}$ for semi-membranosus muscles is somewhat low, but this may partly be due to less perfect registration of tension on account of the shape of the muscle.

The values in Table I of Tl/H for different durations of tetanus (2, 5, 10 and 20 seconds), as shown by experiments on sartorii of *R. esc.* are very similar,

increasing a little with increase in the duration of stimulus. These results may be plotted in a curve relating H/Tl to duration of stimulus t . The relation obtained (which for short will be called the heat-duration curve) was approximately a straight line, showing that the change in the reciprocal ratio Tl/H with increasing t is entirely determined by the nature of the energy regulation in a prolonged contraction as defined (for all but very short times) by the equation

$$H/Tl = A + Bt.$$

The slope of such a line gives directly the heat in gramme centimetres per unit of tension-length maintained per second of stimulus, which in the case of the sartorii of *R. esc.* at 20.3° C. is 0.93, agreeing very well with the value of 0.92 found at 20° C. by Hartree and Hill 10 years ago with very different instruments. Several experiments, however, at 0° C. similarly plotted gave a slope of 0.11, widely different from the value found by Hartree and Hill, 0.17, at the same temperature. The reason for the discrepancy is as follows. Owing to the fact that the muscle at 0° C. is very much slower than at 20° C. a 2-second tetanus at 0° C. is equivalent only to one of about 0.2 to 0.3 second at 20° C. The heat-duration curve in its very early portion (see fig. 1) has a steeper slope, and to attain true linearity at 0° C. longer durations are necessary. Hartree and Hill stopped at a 2-second tetanus for all temperatures and the slopes at the lower temperatures obtained by them are too great, the more so the lower the temperature. It follows also that the temperature coefficient 2.3, given by them, is too small and requires correction. With this in view a fresh series of experiments on the sartorii of *R. temp.* was carried out at five different temperatures. One muscle pair was used for one temperature only and given a series of tetani of increasing durations from 0.45 second to 7 seconds at 20° C., and from 3 seconds to 21 seconds at 0° C., with intermediate ranges of duration for the intermediate temperatures. With the ranges of duration used in these experiments the heat duration curves are practically straight lines from the beginning. On account of the increased economy with which tension is maintained after a certain amount of previous activity (see next section), there is a tendency, after a series of stimuli, for the curve to flatten out a little towards the end. In finding the slopes the straight portions only are considered. The values obtained, therefore, represent the condition for fresh muscle. Table II gives the slope of the H/Tl duration curves in the various experiments at the different temperatures, and fig. 1 (inset) relates the logarithm of the slope to temperature, giving sufficiently closely a straight line. The mean temperature coefficient is 3.1 (instead of the 2.3 given by

Table II.—Slopes of the lines relating H/Tl to duration of Stimulus in Normal Muscles (*R. temp.*) at various Temperatures.

0° C. 1AA muscle		10° C.		15° C.	20° C.
(0·116)	0·134	0·133	0·252	0·35	0·78
(0·094)	0·103	0·147	0·396	0·56	0·94
(0·104)	0·100	0·214	0·33	0·58	0·97
—	—	0·192	0·22	0·61	0·91
—	—	—	0·27	0·71	0·93
—	—	—	0·33	0·74	—
Mean	0·105	0·112	0·172	0·30	0·59
				0·59	0·91

Hartree and Hill (1921)), with the aid of which the mean slopes at different temperatures may be smoothed and classified as follows:—

Temperature, °C.	0	5	10	15	20	25
Slope	0·094	0·165	0·291	0·513	0·903	[1·59]

A change of temperature may be regarded in this connection as affecting merely the time scale of the contraction and of the associated liberation of energy. When a muscle is stimulated for 1 second at 20° C. it will give just the same ratio H/Tl as when stimulated for 3·1 second at 10° C. or for 9·6 seconds at 0° C. An actual time of stimulation t at any given temperature θ corresponds to a certain "reduced" time t_0 at 0° C., the relation being simply $t_0 = t(3·1)^{\theta/10}$, the number 3·1 being the temperature coefficient for 10° C. In fig. 1 all the experiments performed at temperatures from 0° to 20° C. are shown together by the device of plotting H/Tl *not* against the actual time t of stimulation, but against the "reduced" time t_0 . We see that they lie very consistently upon or near a single line which becomes straight after a "reduced" time of about 6 seconds. To find the value of H/Tl for any time of stimulation t at any temperature θ all we have to do is to calculate

$$t_0 = t(3·1)^{\theta/10},$$

and to look up H/Tl for time t_0 in fig. 1. The isometric time coefficient $K_{s(H)}$ can immediately be calculated by dividing t by H/Tl.

By such means it is possible to compare the relation of tension to heat with that of tension to lactic acid production (or phosphagen breakdown). It is necessary merely to know the value of the caloric quotient (heat per gramme of lactic or phosphoric acid) in order to pass directly from $K_{s(H)}$ to $K_{s(L)}$ or $K_{s(P)}$. Conversely from a knowledge of the heat coefficient and the lactic acid (or

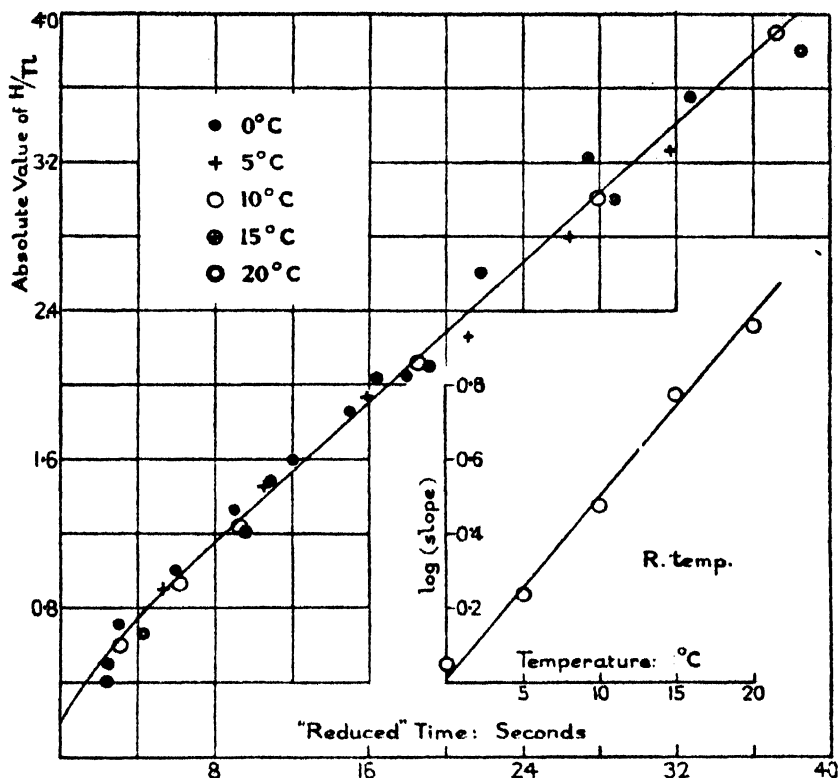


FIG. 1.—Absolute values of H/Tl at five different temperatures, plotted against duration of stimulus expressed in terms of "reduced" time, defined by the equation, $t_0 = t(3.1)^{\theta/10}$, where t is the actual duration of tetanus at any temperature θ and 3.1 is the mean temperature coefficient. Each point observed is the average taken from several experiments at the temperature in question. Note that for all temperatures the points lie on or near the same curve and that the curve becomes a straight line after about 6 seconds "reduced" time. It can safely be extrapolated for a single tetanus of a fresh muscle for as long as 20 seconds at 20° C., or to a "reduced" time of about 200 seconds. For convenience in calculation the slope of the curve may be taken as 0.004 over its linear portion and the following table of "reducing" factors may be used:—

Temperature ° C.....	1	2	3	4	5	6	7	8	9
$(3.1)^{\theta/10}$	1.12	1.25	1.40	1.57	1.76	1.97	2.21	2.47	2.77
Temperature ° C.....	10	11	12	13	14	15	16	17	18
$(3.1)^{\theta/10}$	3.1	3.47	3.89	4.36	4.87	5.46	6.11	6.82	7.68
Temperature ° C.....	19	20	21	22	23	24	25		
$(3.1)^{\theta/10}$	8.6	9.6	10.8	12.0	13.5	15.1	17.0		

Inset.—The logarithm of the slope of the curve averaged for each temperature plotted against the temperature, to give the mean temperature coefficient 3.1.

phosphagen) coefficient it is possible to deduce the corresponding caloric quotient. Take, for example, the recent determinations by Meyerhof and Schulz (1931) of $K_{z(L)}$, in which the lactic acid measured includes that which is delayed in its appearance. This coefficient is the ratio of Tl to the *total* lactic acid which appears as the result of stimulation. It might be objected that the heat determined in the present investigation and shown in fig. 1 is initial heat, while total anaerobic heat is required. The delayed anaerobic heat, however, unlike the delayed lactic acid, is only a small fraction of the whole—probably about 10 per cent.—and a correction can be applied if wanted by adding 10 per cent. to the heat read off from fig. 1 before calculating $K_{z(H)}$.

At 4° C., for example, Meyerhof and Schulz give (1931, Table IV) $K_{z(L)} = 120 \times 10^4$ for a 4 to 6 second tetanus. Multiplying this by 0.69* to correct for the oblique fibre arrangement of the gastrocnemius we obtain $K_{z(L)} = 83 \times 10^4$. A tetanus of 5 seconds at 4° C. is equivalent to one of $5 \times (3.1)^{4/10} = 7.85$ seconds at 0° C., for which, from fig. 1, $H/Tl = 1.15$. Adding 10 per cent. to this to allow for the delayed anaerobic heat we get $H_{total}/Tl = 1.27$. Thus $K_{z(H)} = 5/1.27 = 3.94$. Hence the caloric quotient is $83 \times 10^4/3.94$ g. cm. or $83 \times 10^4/3.94 \times 42650 = 492$ calories per gramme of lactic acid. At 18° C. Meyerhof and Schulz (1931, Table VI) give $K_{z(L)} = 36 \times 10^4$ for a 1 to 2 second tetanus, which, corrected for the oblique arrangement, is 25×10^4 ; 1.5 second at 18° C. is equivalent to $1.5 (3.1)^{1.5/10} = 11.5$ seconds at 0° C.; for which, from fig. 1, $H/Tl = 1.49$. Adding 10 per cent. to allow for delayed anaerobic heat we have 1.64, so that $K_{z(H)} = 1.5/1.64 = 0.915$. Hence the caloric quotient is $25 \times 10^4/0.915 \times 42650 = 636$ calories per gramme of lactic acid. These high values, 492 calories and 636 calories respectively, of the caloric quotient for lactic acid are easily intelligible, since in such short tetani a large part of the energy is supplied, not by lactic acid formation, but by phosphagen breakdown. Another result given by Meyerhof and Schulz (1931, Table VIII) is not so readily explained. At 18° C. they give for a 10 second tetanus the value 69×10^4 , or corrected for fibre arrangement, 47.5×10^4 . The "reduced" time is $10 \times (3.1)^{10/10} = 76.7$ seconds. Assuming that for longer times the line in fig. 1 remains straight the value of H/Tl for 72.2 seconds is approximately 7.1. Adding 10 per cent. we obtain 7.8. Thus $K_{z(H)} = 10/7.8 = 1.28$. Hence the caloric quotient is $47.5 \times 10^4/1.28 \times 42650 = 870$ calories per gramme of lactic acid. It is difficult to see how the caloric quotient can be so high in a long tetanus in which presumably the greater part of the energy is supplied by lactic acid formation. We should expect it to be about 400 calories.

The curve of fig. 1 could be usefully employed, in conjunction with a series of simultaneous determinations of lactic acid and phosphagen in an isometric tetanus, to determine the true caloric quotients of both. Let Q_L be the heat

* Added June 27, 1931.—This number is probably too great. Recent direct measurements by Professor A. V. Hill of the ratio Tl/H in the twitches of a gastrocnemius have given a value of about 11 or 12, whereas for the sartorius it is about 5. The factor must be 0.4 to 0.5. This will somewhat alter the calculations given: for example, with a factor of 0.45 instead of 0.69, 870 calories will become 567 calories—a more reasonable figure.

due to the liberation of 1 g. of lactic acid, Q_L that due to the liberation of 1 g. of H_3PO_4 from phosphagen. Let H be the heat and Tl the tension-length, L the lactic acid set free and P the phosphagen broken down, in the same contraction. Then, denoting the first experiment by suffix 1,

$$L_1 Q_L + P_1 Q_P = H_1.$$

Now, given the time of stimulation, the temperature, and the observed value of Tl , H can immediately be calculated from fig. 1. We have, therefore, two unknowns, Q_L and Q_P , and need another equation. This is obtained by stimulating for a very different time, so that the relative values of L and P are altered. We obtain

$$L_2 Q_L + P_2 Q_P = H_2.$$

These two equations can be solved to give the true caloric quotients Q_L and Q_P .

II.—THE CHANGE OF THE TENSION HEAT RELATION WITH THE AMOUNT OF PRECEDING ACTIVITY.

In the experiments on 2-second stimulation given in section 1 of this paper successive 2-second tetani were usually given in nitrogen at intervals of about 3 minutes until the muscle was nearly exhausted. For the averages of Table I only the first one or two observations in each series of such experiments were used. This was quite necessary, since the ratio Tl/H increased appreciably in the course of the series. This change in the "economy" of maintenance of tension is clearly shown in fig. 2 below, where the values of Tl/H in successive 2-second tetani are plotted against the amount of heat liberated in all preceding activity. The curves generally show an initial drop which, however, is usually small, and it is doubtful if much significance is to be attached to it. The rise in the later part of the curve is a regular feature.

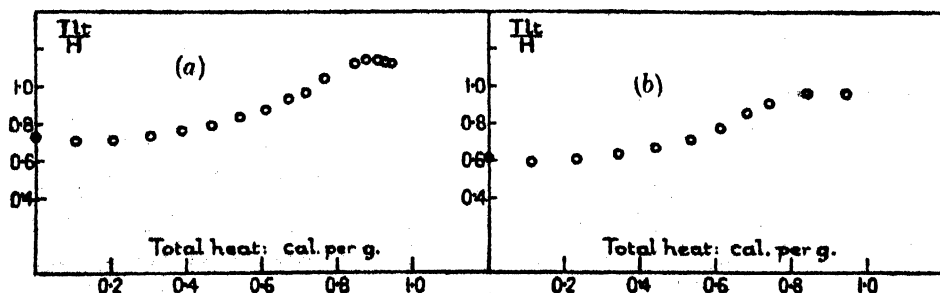


FIG. 2, a and b.—Change of $K_{s(H)}$ with amount of preceding activity, the latter being measured by the amount of heat in calories per gramme previously liberated, in a series of 2-second tetani. Sartorii of *R. sac.* at $20.3^{\circ}C$.

In the series of 2-second tetani studied, an average rest interval of about 3 minutes was interposed between successive observations. During this time anaerobic recovery, in the sense of phosphagen resynthesis at the expense of delayed lactic acid formation, must be going on. It was to be expected that the rise in the curve would be more striking if such rest periods were eliminated altogether. Experiments with continuous stimulation lasting for several minutes were made to test this. Since at 20° C. it is not easy to get a muscle to maintain its full tension, even in a 20-second tetanus, recourse was had to lower temperatures. At room temperatures, 13° to 15° C., with a rate of stimulation barely sufficient to produce initially a complete tetanus, tension may be quite well maintained for about 1 minute, while at 0° C. half of the tension initially developed may be still maintained after 5 minutes continuous stimulation.

The sartorii of *R. temp.* were able to maintain tensions longer than those of *R. esc.*, at any rate in February, and the former were employed in most experiments of this series.

Nine experiments at 13° to 15° C. with 2 minutes and three experiments at 0° C. with 10 minutes of stimulation were carried out. In each case the total heat was recorded by reading the deflection at 6 second or 12 second intervals, and analysed by a 6 or 12 second heating control by the method described in detail by Hill (1929). The tension record was divided into 6 or 12 second blocks as required and the mean value for each block was measured.

One technical detail may be of interest. The heat production to be measured is large, and in order to keep the spot of light on the scale the recording system has to be made rather insensitive. For sharp analysis, however, the galvanometer must be critically but not over-damped, and the response of the whole recording system must be kept as quick as possible. Low sensitivity must be combined with rapidity of response. This is achieved by the simple procedure of interposing a resistance between thermopile and galvanometer and shunting the former instead of the latter, allowing only the desired fraction of the current developed to pass through the galvanometer. By this means the damping of the galvanometer is not increased and its speed of movement is unaffected.

The results of four such experiments are shown in fig. 3, two at room temperature and two at 0° C., the average tension during each interval being plotted on the tension curve, the total heat in each interval being given as a block in the heat curve. In fig. 4, *a* and *b*, corresponding to the experiments in fig. 3, *a* and *b*, are curves relating $Tl\Delta t/\Delta H$ to the total heat produced in all preceding activity, up to the middle of the interval in question. Here Tl is the average

tension-length maintained during the interval Δt in question and ΔH is the heat liberated in that interval. After the first interval the ratio is obviously

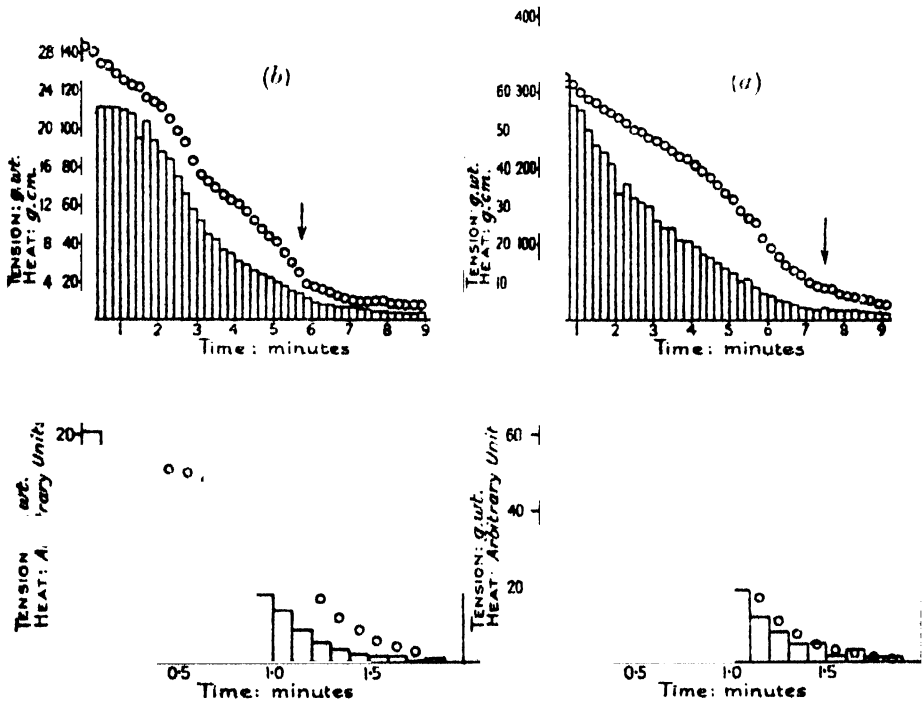


FIG. 3, *a* and *b*.—Sartorius of *R. temp.* at 0° C. 10 minutes continuous tetanus. Heat analysed in units of 12 seconds. Heat in each interval shown by a vertical block; mean tension in each interval by a circle. At the point indicated by the arrow, heat and tension both became small and their measurement uncertain. *c*, Sartorius of *R. temp.* at 13° C. 2 minutes tetanus. Analysis in 6-second units. *d*, Same at 15° C.

independent of the heat liberated in the process of developing, as distinguished from maintaining, tension. In all cases, after an initial constant phase lasting to about half exhaustion, there is a striking increase in the economy of maintenance of tension, which at 0° C. may be as great as fourfold. At the point shown by the arrows in fig. 3 the muscles had become practically exhausted and the ratio of tension to heat become rather indeterminate, tending, however, to decrease somewhat owing to the failure of the muscle. The curves of fig. 4, *a* and *b*, are not continued beyond the arrows shown in fig. 3, *a* and *b*. These results confirm and extend those obtained when the muscle was given a series of 2-second tetani with intervals between.

It is well known that as a muscle is fatigued it becomes slower. This slowing

in the response of the muscle was evident in the tension records of most experiments. Thus the last few of a series of 2-second tetani usually showed a

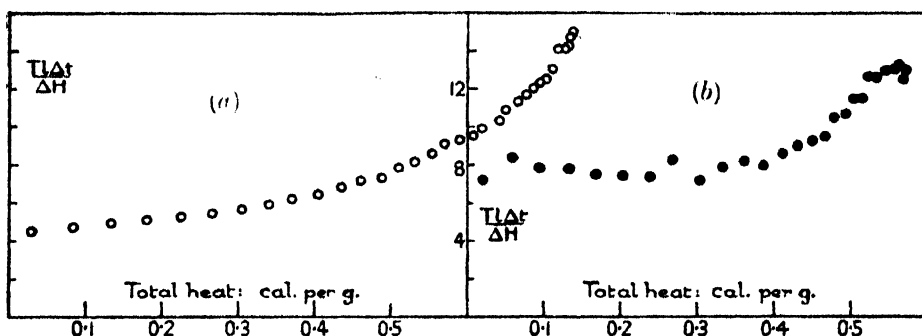


FIG. 4. *a* and *b*.—The results of fig. 3, *a* and *b*, plotted to show the change of $T\Delta t/\Delta H$ with the amount of preceding activity, as measured by the heat in calories per gramme liberated up to the middle point of each interval. The heat analysis was in units of 12 seconds. T is the mean tension exerted during any given interval; Δt is the length of the interval (12 seconds); ΔH is the heat liberated in the interval. 10 minutes continuous tetanus at 0°C .

spreading out of the falling limb of their tension curves. In a long-continued tetanus little teeth which may appear on the record during the early portion of the tension curve, due to just not complete fusion of individual twitches, gradually disappear. There is no doubt that this slowing of the muscle with the onset of fatigue is the cause of the increasing economy observed. The same phenomenon has already been discussed by Bronk (1930) for the case of frogs' muscles and by Bozler (1930) for the case of the retractor of the pharynx of the snail. In the latter muscle the increased economy of maintaining tension shows itself much earlier and reaches a much higher degree.

III.—THE EFFECTS OF POISONING WITH IODO-ACETIC ACID AND OF CURARISATION ON THE TENSION HEAT RELATION IN A TETANUS.

This group of experiments was made on the sartorii of *R. esc*. Heat was measured by the maximum deflection method as in section 1 above. Iodo-acetic acid and trimethyl-octyl-ammonium iodide were administered by soaking the isolated muscle, while curare was usually first given by injection and then again by soaking when the muscle was mounted in its chamber. Table III gathers together the results of the experiments made at 20.3°C . For curarised muscles and for muscles treated with the ammonium salts no control observations were taken, in the sense that the values of $T\Delta t/H$ were not

Table III.—Values of the Isometric Time Coefficient for Heat in Muscles treated with Trimethyl-octyl-ammonium Iodide, with Curare and with Iodo-acetic Acid. All 2-second tetani at 20·3° C.

Condition.	Date of experiment.	T ₄ /H average.	Remarks.
0·08 per cent. AmI for 30 minutes	January 20 (No. 1)	0·75	Inability to maintain tension evident.
0·05 per cent. AmI for 40 minutes	January 20 (No. 2)	0·75	Inability to maintain tension evident, though not so marked as No. 1.
0·04 per cent. AmI for 30 minutes	January 19 (No. 1)	0·85	Impaired ability to maintain tension barely perceptible; doubtful whether muscle really poisoned.
0·08 per cent. AmI for 30 minutes	January 19 (No. 2)	0·65	Very pronounced inability to maintain tension.
12 mg. curare per 100 c.c. for 85 minutes	January 21 (No. 1)	0·86	Tension well maintained.
Injected 1 c.c. 0·25 per cent. curare and subsequent soaking in 18 mg. per 100 c.c. solution	January 22 (No. 2)	0·78	Tension well maintained.
Injected 1·5 c.c. 1 per cent. curare	January 23	0·86	Tension well maintained.
1/16700 IAA, 43 minutes....	January 5 (No. 2)	0·83 (0·82)	Numbers in brackets below the T ₄ /H ratios for IAA muscles are control ratios before introduction of IAA.
1/16700 IAA, 42 minutes....	January 5 (No. 1)	0·66 (0·65)	
1/16700 IAA, 43 minutes....	January 2 (No. 1)	0·58 (0·57)	
1/16700 IAA, 43 minutes....	January 1 (No. 2)	0·87 (0·87)	

measured previously on the same muscle. The values found, however, are clearly within the normal range of variation. In muscles poisoned with iodo-acetic acid controls were made, the ratio before and after iodo-acetic acid being practically the same. This result confirms Fischer (1931), who found T/H for a 0·6-second tetanus to be unchanged in an iodo-acetic muscle. On iodo-acetic muscles experiments were also made at 0° C. to obtain the slope of the heat-duration curve. They give the same slope as normal muscles. For convenience these results have been included in Table II. It may be worthy of note that a higher iodo-acetic acid concentration is required to poison a muscle at 0° C. than at 20° C. for a given duration of soaking. With the same concentration the period of soaking must be considerably lengthened, more than one would expect from the effect of temperature on the velocity of diffusion, so presumably some reaction of the substance with the muscle is involved.

Muscles even heavily curarised maintain tension in a long tetanus at least as perfectly as normal muscles; sometimes they give the impression of being better. The same fact was noticed by Hartree and Hill (1921). Muscles poisoned with the ammonium salt, on the other hand, while giving a normal T_k/H , provided a very poor tension record. During a single 2-second tetanus the tension fell progressively, although initially it was of normal size. Nachmannsohn (1929) observed that muscles poisoned by soaking in the ammonium salt solution were less able to maintain tension than when the substance was injected. I have not been able to verify this observation. It appears that the effect of trimethyl-octyl-ammonium iodide in this respect is variable. In the majority of Nachmannsohn's experiments it would seem that the poisoned muscles maintained tension well, while in none of mine was this the case. Recently Meyerhof and Schulz (1931), repeating Nachmannsohn's experiments, also found the impaired ability of the muscles poisoned with ammonium salts in respect of tension maintenance.

According to Nachmannsohn (quoted from Meyerhof, 1930, p. 236), $K_{z(L)}$ for muscles treated with trimethyl-octyl-ammonium iodide or curare is about the same as for normal muscles; if anything, that for the curarised muscle is higher— 70×10^6 as against 50×10^6 . His results are now open to the objection that no attention was paid to the delayed lactic acid, of which a variable amount must have been included in his experiments. For phosphagen breakdown in such muscles $K_{z(P)}$ has been redetermined by Meyerhof and Schulz (1931) and found for both to be approximately double the normal value. An abnormally high $K_{z(P)}$ may be due either to an actual restriction of phosphagen breakdown or to a quickened resynthesis following decomposition. The decision between the two alternatives is of great importance in our comprehension of the mechanism of muscular contraction. Unfortunately there is no direct basis for judgment. In either case, however, in order that $K_{z(H)}$ in curarised muscles should be unchanged—as found above—their $K_{z(L)}$ should be smaller than usual, unless a third chemical reaction of energetic importance complicates the situation. A redetermination of $K_{z(L)}$ for such muscles is highly desirable.

For an iodo-acetic muscle, on the assumption that the heat liberated is all derived from phosphagen breakdown, we can make a rough calculation of the $K_{z(H)}$ from chemical data for comparison with that observed. 1 g. of H_2PO_4 split from phosphagen may be assumed to give 160 calories (Meyerhof, Lunds-gaard and Blaschko, 1931). The average of four values of $K_{z(P)}$, at about $15^\circ C.$, for a single 2-second tetanus in an iodo-acetic acid treated gastrocnemius, given

by Lundsgaard (1930) is 12×10^6 . This gives, corrected for the oblique fibre arrangement of the gastrocnemius,

$$Tlt/H = 12 \times 0.69 \times 10^6 / (160 \times 42400) = 1.22.$$

The value of $K_{z(H)}$ deduced from fig. 1 for a 2-second tetanus ("reduced" time = 10.9 seconds) is 1.40. Since in Lundsgaard's experiments the temperature was not strictly controlled but probably somewhat below 15° C. (personal information), the agreement is satisfactory.

IV.—A NOTE ON THE EFFECT OF FREQUENCY OF STIMULATION ON THE VALUE OF T/H IN A TETANUS.

It was shown by Hill (1913) that the heat produced per unit of tension developed and maintained in a tetanus is independent of the frequency of stimulation, provided that the latter be sufficient to cause a complete tetanus. This was fully confirmed in a number of experiments made with the present myothermic technique. Three such experiments on a 12-second tetanus at 0° C. are shown in fig. 5 (note that experimental variations are exaggerated by

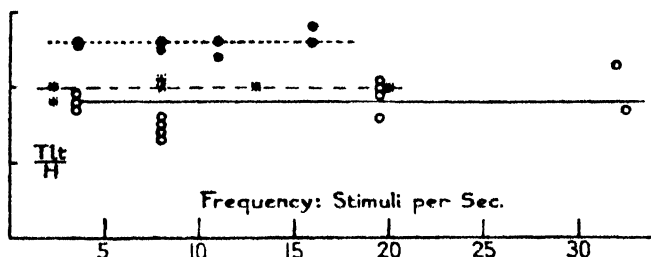


FIG. 5.—Three experiments on sartorii of *R. temp.* at 0° C. with 12-second tetani, at various frequencies of stimulation, showing that $K_{z(H)}$ is independent of frequency.

plotting on a large scale; the zero line is not given). In view of this result, in the experiments reported earlier in this paper no attention had to be paid to the frequency of stimulation, provided that the latter was not too low for complete fusion or so high that failure of excitation occurred in the course of a contraction.

Summary.

(1) The heat-duration relation has been redetermined for frogs' muscle undergoing a maximal tetanic stimulus of suitable frequency. H being the initial heat, T the mean tension developed and maintained, l the length of muscle, and t the duration of stimulus, H/Tl has been determined as a function of t for five different temperatures from 0° to 20° C. The relation soon becomes

linear, and the mean temperature coefficient for 10° of the final slope is found to be 3.1 instead of the 2.3 given by Hartree and Hill (1921). Their value is too low owing to their use of too short tetani for the lower temperatures.

(2) The effect of temperature in this connection may be regarded merely as an alteration in the time scale of muscular contraction. A duration of tetanus t at any temperature θ is equivalent to another "reduced" duration t_0 at 0° C., where $t_0 = t(3.1)^{\theta/10}$. If H/t at any temperature be plotted not against the actual duration of stimulus but against the "reduced" duration, the plotted points all lie on or around a single curve which becomes a straight line after a "reduced" time of about 6 seconds. This curve, apart from its theoretical interest, enables one to deduce the value of the isometric time coefficient for heat ($K_{z(H)}$) at any temperature for any duration of tetanus. Examples of this deduction are given.

(3) By dividing Meyerhof's isometric time coefficient for lactic acid $K_{z(L)}$ by $K_{z(H)}$, a value of the caloric quotient for lactic acid can be obtained in gramme centimetres per gramme of lactic acid. Examples of the calculation are given. A method is suggested by which the true caloric quotients for lactic acid and phosphagen could be determined.

(4) The ratio t/H increases progressively from the stage when the muscle is about half exhausted, whether it is given a series of short tetani with rest intervals between them or is stimulated continuously. This increase in "economy" is due to the progressive slowing of the muscle as fatigue sets in.

(5) Muscles poisoned by iodo-acetic acid, by curare, or by trimethyl-octyl-ammonium iodide, all give normal values of $K_{z(H)}$. The comparison of $K_{z(H)}$ experimentally determined with that calculated from the observed $K_{z(P)}$ in an IAA muscle gives satisfactory agreement.

(6) Hill's observation that t/H is independent of the frequency of stimulation, provided that the latter be not too low to produce complete fusion and not so high as to cause failure of excitation, is confirmed.

I am deeply indebted to Professor A. V. Hill who suggested the work, and under whose kind direction and help it was carried out. To Mr. J. L. Parkinson I wish to express my thanks for much skilful assistance.

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The Integration of Plant Behaviour. IV.—Geotropism and Growth-Substance.

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Introduction.

In a previous paper (1929) we referred to an ingenious experiment by Cholodny (1924), who, working with *Zea Mays*, found that the sensitivity to gravity of decapitated roots can be largely restored not only (as was already known) by replacing their own tips, but also, and even better, by placing on them the tips of coleoptiles. Whichever tip was placed upon them, the roots curved downwards. Since decapitated coleoptiles also are known to have their sensitivity to gravity restored if their tips are replaced, and since they respond by curving upwards, the question at once suggests itself—how is it that the coleoptile tip causes the stumps of coleoptile and root to respond to gravity by curving in opposite directions? It seems clear that the difference in the responses must somehow depend upon the different natures of the stumps, and Cholodny considers that these are so constituted that their growth rates are oppositely affected by the same substance which diffuses into them from the coleoptile tips, the growth of the coleoptile being accelerated and that of the root being retarded. Stark also (1927, p. 8) adopts a view which in this respect is similar.

Naturally in order to explain the curvatures along these lines, it is necessary to suppose further that this substance for some reason flows to the lower sides of the horizontal stumps in greater concentration than to the upper sides. That a substance accelerating the growth of the coleoptile of *Avena* really does diffuse out of its tip had been made very probable by Söding (1925) and Went has since rigorously established the fact (1928).

Cholodny supports his theory by finding that the rates of growth of decapitated roots and coleoptiles are affected in opposite senses if either root tips or coleoptile tips are placed upon them, the roots being retarded by both kinds of tip and the coleoptiles being accelerated by both kinds (1924, 1926 and 1928). We have been led on to investigate for ourselves the effects of the two kinds of tip on the growth of the root, after first confirming Cholodny's original discovery.

Experiments.

As a first step, the downward curvatures of maize roots, decapitated at 1 mm. from the end of the root cap, re-headed with their own tips or with coleoptile tips, and placed horizontal, were compared with the curvatures of similarly decapitated controls and of intact roots. The results are shown in the following table. The curvatures given are the means amongst the total number of roots in each series.

Table I.—*Zea Mays.*

	Number of roots.	Mean curvature after—		Percentage of roots that curved.
		5-6 hours.	20-24 hours.	
Intact roots	41	56	54.6	100
Decapitated roots re-headed with coleoptile tips	129	18.9	33.2	82.9
Decapitated roots re-headed with root tips	58	9.1	17.1	58.6
Decapitated controls	192	—	2.3	7.2

The curvatures reported were all directed downwards, and therefore geotropic. Only a very few roots showed accidental curvatures in a horizontal plane, and not a single root in the above series curved upwards.

These results fully confirm Cholodny's discovery that coleoptile tips cause root stumps to curve strongly downwards, and that they do so more effectively than replaced root tips. But the root tips also caused the stumps to curve

downwards fairly well in comparison with the decapitated controls, which showed very few curvatures. The coleoptile tips did not make the root stumps curve as strongly as intact roots. Whichever tip was used, the re-headed roots not only curved less than the intact roots, but usually began to curve much later. A large proportion of them did not begin to curve until after the first 5 or 6 hours, whereas all the intact roots had curved strongly within this time. After from 20 to 24 hours the mean curvature of the intact roots was slightly less than after 5-6 hours. It is indeed well known that roots in damp air usually do not curve down so far as the vertical, but grow on at an oblique angle.

In the series of roots re-headed with coleoptile tips, the stumps or the tips or both were often washed in running water for about 15 minutes, in order that any wound substances might be removed from the cut surfaces. Also many of the decapitated controls were similarly washed. But washing did not seem to make any difference to the curvatures.

Other maize roots were decapitated at 2 mm. from the end instead of 1 mm. and re-headed with coleoptile or root tips, and placed horizontal. Many curved downwards, but the results were distinctly less good than when the roots were decapitated at 1 mm. It may be noted that Cholodny decapitated at from 1 to 1.5 mm. from the end (1924, p. 356). The length of the root cap in maize is about 0.5 mm.

In another experiment, 28 maize roots were decapitated at 1 mm., re-headed with coleoptile tips that had been killed in boiling water, and placed horizontal. Only two curved, their curves reaching 25° and 30° downwards. Also 23 maize roots similarly decapitated were re-headed with similarly killed root tips. Only one curved down, the curve reaching 50°. The proportion of curvatures with dead tips (altogether 3 in 51 roots) was slightly less than was found in the decapitated controls of Table I. Dead tips therefore do not cause the root stumps to curve geotropically.

Since, therefore, the living coleoptile tip makes the root stump curve downwards, whereas it makes the coleoptile stump curve upwards, the next point was to determine whether it also affects the growth of the root differently from that of the coleoptile. For this purpose, the growth of roots of *Zea Mays* decapitated at 1 mm. from the end and re-headed with coleoptile tips, was compared with that of similarly decapitated controls. Pairs of roots were used, in this and in subsequent experiments, which had been growing at nearly equal rates beforehand, and in other respects also the methods were those which we described previously (1931). The following table shows the growth of the

re-headed roots in each series, expressed as a percentage of that of the controls with which they were paired :—

Table II.—*Zea Mays*.

Number of pairs.	Growth of roots re-headed with coleoptile tips in first 5-6 hours.	In next 16-18 hours.
11	95.5	98.5
10	100	59
10	78	73
10	62	87
Total 41	Mean 84.1	Mean 79.8

From these results, it appears that, as Cholodny has found (1924) the coleoptile tip does indeed retard the growth of the root stump, although it is known to accelerate the growth of the coleoptile stump in the oat (Söding, 1925), and also, according to Cholodny (1924, p. 362), in maize.

In the coleoptile of *Avena*, the tip, if replaced eccentrically, on one side of the stump, causes a curvature towards the opposite side (Nielsen, 1924). This is according to expectation, since the tip accelerates growth in the parts of the coleoptile directly beneath it. We, therefore, performed the experiment of placing coleoptile tips of maize eccentrically upon root stumps, as in fig. 1.



The roots were decapitated at 1 mm. from the end and were kept vertical. After 24 hours, 6 roots out of 12 had curved towards the side covered by the tip, the curves being mostly of about 20°, but one of them being of 50°. The other six roots remained straight.

Also on 24 other similarly decapitated maize roots coleoptile tips were placed eccentrically, as before, but the root stumps were then placed horizontal, and in such a way that they and the tips were in the same horizontal plane. Some of the roots curved obliquely downwards, but the horizontal components of the curvatures, after 24 hours, were directed towards the side covered by the tip in 13 of the roots and towards the other side in one only. The remaining 10 roots showed no horizontal curvature. These experiments, therefore, also indicate that the coleoptile tip retards the growth of the root.

Since, therefore, the coleoptile tips affect in opposite senses the rates of growth of the stumps of coleoptile and root, it becomes less surprising that they also cause these stumps to curve in opposite directions. But it has also

to be considered how it is that the root stumps are made to curve in the *same* direction by the tips of coleoptile and root. It might be expected that the two kinds of tip would be found to affect in the same sense the rate of growth also of the root stump. The coleoptile tip has already been shown to retard the growth of the root, and the next question therefore is what is the effect on growth of the root tip. Cholodny has found that the growth of decapitated roots of *Lupinus angustifolius* (1926, p. 458, and 1929, p. 473) is retarded if their tips are replaced upon them. But in his earlier experiments, the retardation was slight, and in the later ones, the number of roots tested was rather small, so that it seems worth while to submit further evidence on the point. He has also found that root tips of *Zea Mays* accelerate the growth of decapitated coleoptiles of *Avena* (1928, p. 126, and 1929, p. 471), and increase their sensitivity to geotropic and phototropic stimuli (1928, p. 123, and p. 125), so that in all these respects root tips appear to act similarly to coleoptile tips, though probably less strongly.

In order to investigate the effect of the root tip on the growth of maize roots, we compared the growth of roots decapitated at 1 mm. from the end with that of similarly decapitated roots, on which the tips were replaced. The roots were kept vertical. At first we obtained puzzling and rather irregular results ; for in most of the series, the re-headed roots grew slightly the faster, though in some of the series they grew slightly more slowly or about equally, as the following table shows.

Table III.—Growth of Decapitated Maize Roots re-headed with their own Tips expressed as a percentage of that of similarly Decapitated Controls.

Number of pairs.		Growth in first 5-6 hours.	In next 16-18 hours.
11		113.3	108.2
7		121	126
7		113	110
8		113	104
5		115	102
10		100	100.5
6		107.1	50
12		95.4	97.2
5		92.3	146.6
Total	71	Mean 107.1	Mean 104.0

Since, therefore, these results were puzzling, it occurred to us that they might have been somehow complicated by the shock of the wound. We, therefore,

arranged another experiment that was similar to the last except that after decapitation the roots that were to be re-headed, and also the control roots, were left for 6 hours to recover from the shock. Then the tips of a third set of roots were cut off at 1 mm. from the end and placed upon one of each pair of the previously decapitated roots, and the measurements were started. The results are given in the following table.

Table IV.—Growth of Maize Roots re-headed with Tips of other roots 6 hours after Decapitation, as a percentage of that of similarly decapitated Controls.

Number of pairs.		Growth in 18 hours.	
	5		96.6
	5		52.6
	10		83.3
	9		98.3
	10		67.7
	5		88.2
	9		79.2
	6		77.0
Total	59	Mean	76.0

In another series of 11 pairs, the decapitated roots were left for 18 hours before tips were placed on one of each pair. In the next 6 hours the growth of the re-headed roots was 71.4 per cent. of that of the controls, and in the next 18 hours again it was 64.7 per cent.

In all these nine series, therefore, the re-headed roots grew the more slowly, and in most of them very considerably so. These results, therefore, show that the root tip does indeed retard the growth of the root.

This conclusion is supported by the following experiment, in which tips of maize roots cut off at 1 mm. from the end, were placed on the stumps eccentrically, just as in the corresponding experiment with coleoptile tips (fig. 1). The roots were kept vertical. After 24 hours, out of 24 root stumps thus treated, nine had curved towards the side covered by the tip—most of them in fairly strong curves of from 30° to 40°, and only one had curved in the opposite direction, in a curve of about 25°. The remaining 14 were still straight.

In another series of six roots, in which the stumps and tips were washed for 15 minutes before the tips were placed on the stumps, after 6 hours three had curved towards the side covered by the tip, in curves of from 18° to 35°, and three remained straight.

Also previously (Snow, 1923), root tips of *Vicia faba* were cut off and replaced eccentrically. After 24 hours, four of the stumps had curved slightly towards the side covered by the tip and five were still straight.

Root tips were also placed eccentrically on 30 other decapitated maize roots, as before, but the roots were then placed horizontal, in such a way that they and the tips were in the same horizontal plane. Some of the roots curved obliquely downwards, but the horizontal components of the curvatures, after 24 hours, were directed towards the side covered by the tip in 13 of the roots, and towards the other side in one only. The remaining 16 showed no horizontal curvature.

In another experiment the tips removed from 30 young maize roots were cut in half in a median plane, and a half tip was then replaced on one side of each stump, in the manner shown in fig. 2, in the vertical position. After 24 hours, six of the roots had curved towards the side covered by the half tip and one had curved the other way. The remaining 23 were straight. Eleven roots of *Vicia faba* also were treated in the same way. After 24 hours, 6 had curved towards the side covered by the half tip. The remaining 5 were straight.

In all of these experiments with root tips, therefore, the curvatures were all or nearly all directed towards the side covered by the tip (or half tip), just as in the experiments with coleoptile tips. The most probable explanation is again that the tips retarded the growth of the side of the root covered by them, and these experiments therefore provide strong evidence to show that root tips act in the same sense as coleoptile tips, and retard the growth of root stumps.

FIG. 2

The most obvious way of investigating the effect of the root tip on growth is to compare the growth of decapitated roots with that of intact roots, an experiment which in the past has given very conflicting results. We performed this experiment many times on maize roots, but the results did not help towards determining the effect of the tip on growth, on account of the effect of the wound-shock, which seemed quite to obscure any effect due to loss of the tip.

The results reported in this paper confirm those of Cholodny, who found that coleoptile tips retard the growth of root stumps, though they accelerate that of coleoptile stumps. They also strongly support his discovery that the root tip also retards the root stump, though in the first experiment on this point the results were irregular and partly conflicting, apparently because in some way not understood they were complicated by the wound shock. It therefore appears that the growth rates of root and coleoptile are indeed affected in

opposite senses by the same substances, and this conclusion is further supported by interesting experiments of Nielsen (1930), who has found that a substance excreted by a certain fungus strongly accelerates the growth of coleoptiles and strongly retards or even arrests that of roots. An experiment by Hammett (1929, p. 316) might, indeed, appear at first to conflict with this conclusion : for he found that some substances extracted in dilute acid from crushed tips of maize roots *accelerated* the growth of other maize roots. But it is probable that the substances extracted from crushed tips are different from those which diffuse out of uninjured tips ; indeed, Went found (1928, p. 22) that the normal growth-regulating substance of the coleoptile tip of *Avena* could never be extracted from dead or crushed tips.

The theory that the opposite geotropic curvatures of root and coleoptile are due to redistributions of the normal growth substances produced by the tips, which accumulate in greater concentration on the lower sides of the responding zones, appears very probable. Certain objections to the theory have indeed been raised with regard to the relative efficiencies of the two sides of the stump in transmitting the stimulus, but discussion of these must be reserved for another occasion. The most urgent question, as Cholodny points out, appears now to be what is the change set up in the stimulated tissues which leads to the redistribution of the growth substance. In a previous paper (1929, p. 494) we made it clear that the redistribution must be supposed to take place partly in the tip and partly in the stump, and Dolk has shown that in horizontal coleoptiles the growth-accelerating substance does actually become redistributed both in the tip (1929, *a*) and also in the parts behind the tip (1929, *b*), in such a way as to reach the lower side in greater concentration.

Summary.

(1) Confirmation is given of Cholodny's discovery that the sensitivity to gravity of decapitated maize roots is restored by coleoptile tips placed upon them, even better than by root tips.

(2) The growth of decapitated maize roots was found to be retarded by coleoptile tips, and also by root tips provided that the roots were left for 6 hours to recover after decapitation.

(3) When coleoptile tips or root tips or halves of root tips were placed eccentrically upon decapitated roots, either in the vertical or in the horizontal position, the roots curved towards the side covered by the tip.

(4) The results support Cholodny's theory that the opposite directions of geotropic curvature in root and coleoptile are correlated with the fact that the

growth of root stumps is retarded by coleoptile tips or root tips, though that of coleoptile stumps is accelerated by them.

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The Breakdown and Resynthesis of Phosphagen in Frozen and Thawed Muscles.

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E. C. Smith (1929) has described the breakdown of glycogen to lactic acid which occurs in muscle as a result of freezing, and the conditions under which resynthesis may occur in the thawed muscle. The present work covers the same ground in respect of the breakdown and resynthesis of phosphagen, and the behaviour of the hexose phosphoric esters and adenylyl-pyrophosphoric acid. Only in the case of phosphagen have important changes been observed.

As in the case of glycogen, breakdown of phosphagen occurs in the muscle merely as a result of freezing. The rate of breakdown increases to a maximum at -2.6° , the identical temperature at which the breakdown of glycogen is a maximum, and in each case the rate of breakdown falls away to a negligibly small value at -10° .

The muscle is capable of recovering to a greater or less degree its

contractility if freezing has not been too severe (Moran, 1929). Thus, after 24 hours at -1.6° recovery of function is complete, but with increasing severity of freezing recovery becomes progressively more difficult to accomplish and the height of the contraction is diminished until after 24 hours at -2.2° the muscle is rendered permanently non-irritable.

Associated with complete recovery of contractility is a capacity to remove by synthesis to glycogen the lactic acid formed during the freezing period. After 24 hours at -1.6° this faculty is lost, and the muscle on thawing continues to produce lactic acid at a great rate. The results quoted below show that muscle behaves very similarly in respect of phosphagen synthesis, but there is one point of difference in that the loss of synthetic capacity is more gradual. For periods of freezing of 24 hours, between -1.6° and -2.5° , a gradually diminishing power of resynthesis is retained, and this power is only finally lost at a temperature of -2.5° .

Experimental.—The method of determination of the several phosphate fractions closely followed that recommended by Eggleton (1929). For each experiment the gastrocnemius muscle of six frogs were used. In series I and II the muscles of the one side gave the resting values for the five phosphate fractions (phosphagen, inorganic phosphate, pyrophosphate, "soluble esters" and "insoluble esters") whilst those of the opposite side were frozen for 24 hours (in series I) or 6 hours (in series II) at different freezing temperatures. After removing the muscles from the body they were kept overnight at zero in moist air to allow them to recover their resting condition. The experimental muscles were then transferred to a watertight vessel immersed in a thermostat containing brine, which was accurately maintained at the required freezing temperature. After standing for 2 hours at this temperature, each muscle was seeded with a small crystal of ice, the period of freezing being taken to commence at this point.

It was found (fig. 1) that the breakdown of phosphagen was so marked that between the temperatures of -2° and -3° little or no phosphagen was left in the muscles after 24 hours' freezing. A shorter time of storage (6 hours, fig. 3) within this range of temperature served to define the point of maximum breakdown, -2.6° C. The increase in inorganic phosphate followed exactly this change in phosphagen concentration (figs. 2 and 3) for the changes in soluble and insoluble esters, and pyrophosphate were of too small an order to influence to any marked extent the inorganic phosphate. The slight changes observed in these fractions were inconstant and irregular, and apparently without significance.

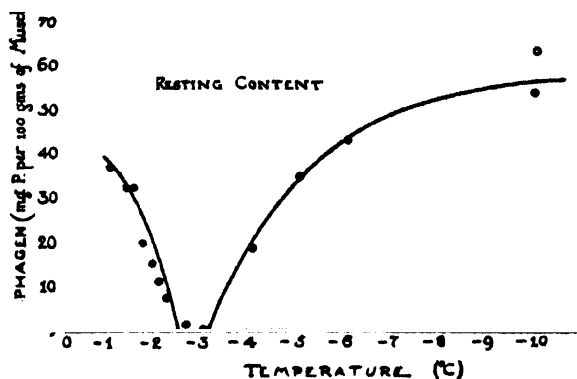


FIG. 1.—Phosphagen content of muscles after 24 hours in the frozen state. Series I.

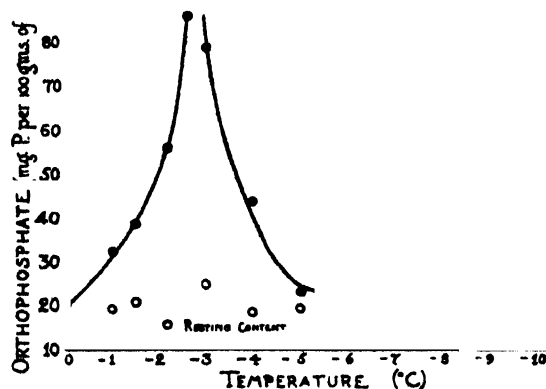


FIG. 2.—Inorganic phosphate content of muscles after 24 hours in the frozen state. Series I.

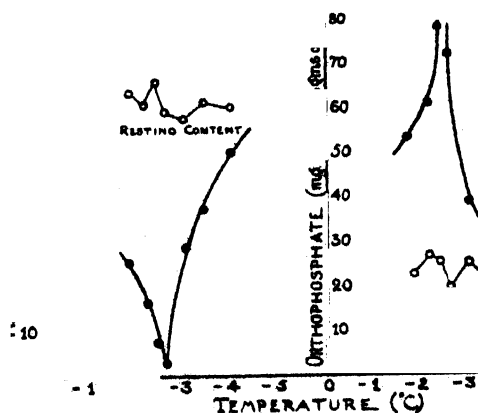


FIG. 3.—Phosphagen and inorganic phosphate content of muscles after 6 hours in the frozen state. Series II.

In a number of filtrates from the frozen muscles lactic acid was determined. The figures obtained fell on the curve of production of lactic acid shown by Smith (1929, fig. 2). The two sets of experiments are, therefore, quite comparable. A comparison of these diagrams makes it quite clear that the breakdown of phosphagen follows an exactly similar course to that of glycogen, and it is highly probable, therefore, that the two reactions are determined by one and the same event in the process of freezing. It is not possible to say whether there is a closer link between the two reactions; whether, for instance, either one of the changes succeeds and is dependent on the other. Some evidence in this connection is given by a study of the behaviour of the muscles on thawing, and the question is further discussed in the paper which follows.

The Recovery of Phosphagen on Thawing.—In the third series both the control and the experimental set of muscles were frozen for 24 hours, the former analysed whilst still frozen and the latter allowed to thaw for 24 hours in an atmosphere rich in oxygen before being analysed. The results are shown in figs. 4 and 5. Muscles frozen for 24 hours at a temperature not less than -2.5° are capable of restoring, to a greater or less degree, their phosphagen

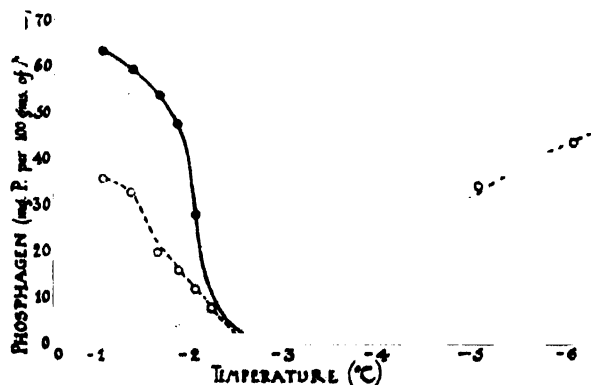


FIG. 4.—Phosphagen content of muscles after 24 hours' freezing (broken line) and subsequent 24 hours' thawing (continuous line). Series III.

content. Above -1.5° the restoration is complete, but below this temperature the phosphagen value reached after 24 hours is less than that of the resting muscles, and becomes progressively less with fall of temperature to -2.5° .

Of the several reasons that might be advanced to account for this lower level of phosphagen in the thawed muscles, the most likely seemed to be that individual muscles in the batch of six worked up together were more tolerant to

freezing than others, and that, if examined singly, muscles would show either complete recovery or complete loss of synthetic power. There was justification

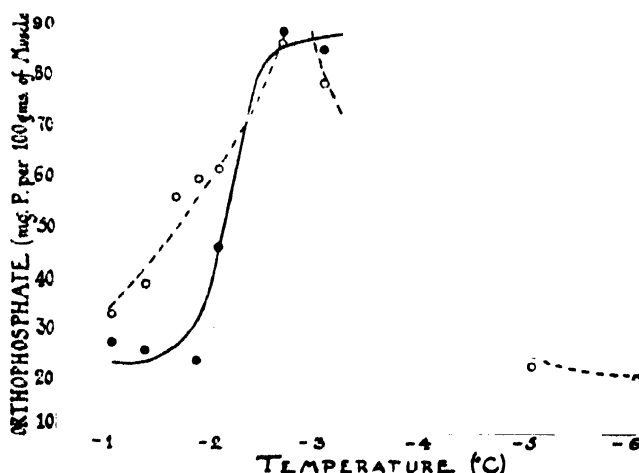


FIG. 5.—Inorganic phosphate content of muscles after 24 hours' freezing (broken line) and subsequent 24 hours' thawing (continuous line). Series III.

for this view on the grounds of analogy with the conversion of lactic acid to glycogen in thawed muscles. However, the experiments summarised in Table I show that single muscles, frozen under standard conditions in the region

Table I.*—Partial Resynthesis of Phosphagen.

Experiment.	Temperature of freezing (° C.).	Phosphagen content (milligram per cent. P).	
		Thawed muscle.	Control.
1	-1.95	48.0	51.5 (unfrozen)
2	-1.95	38.0	50.3 (")
3	-1.95	50.2	57.8 (")
4	-1.95	14.1	Trace (frozen but not thawed)
5	-1.95	27.1	10.3 (")
6	-1.85	24.0	10.0 (")
7	-1.85	38.2	7.1 (")
8	-1.85	50.7	12.2 (")
9	-1.85	4.0	Trace (")
10	-1.85	35.7	10.8 (")
11	-1.85	2.4	Trace (")
12	-1.85	12 hours 11.9 48 hours 13.0 24 hours 29.9 48 hours 10.5	---
13	-1.85		---
		Creatine content of frozen muscle (milligram per cent.).	
14	-2.0	309	
15	-2.0	358	
16	-2.0	412	

* These experiments were carried out by Mr. H. F. Davis.

-1.5° to -2.0° and kept in oxygen for 24 hours after thawing, recover only partially, and to different extents, their phosphagen content. Experiments 12 and 13 show that the time allowed is adequate for recovery to go as far as it will. Further, there is no destruction of creatine during storage in the frozen state (experiments 14-16), so that material for the formation of phosphagen is not lacking.

Either, then, a new equilibrium is set up between phosphate, creatine and phosphagen, or certain individual fibres recover their synthetic capacity whilst in others this function is destroyed. The former alternative seems the less likely. If the latter is true, the gradual diminution of contractility observed by Moran may also be attributed to differential susceptibility of fibres to freezing. There is at present no reason to suppose that contractility is associated with the integrity of the synthetic system in the muscle. A few experiments carried out on similar muscles to those used above showed that contractility is quite marked in muscles frozen for 24 hours at -2.0° , is regained in only a small proportion of muscles frozen at -2.1° , and is completely destroyed at -2.2° . At this temperature at least 10 per cent. of the fibres are capable of synthesising phosphagen, whilst at -2.0° none of the glycogen synthetic power is retained. These three functions appear, therefore, to possess different susceptibilities to freezing.

Fig. 6 shows the results of a number of experiments in which one set of muscles was allowed to thaw in oxygen, whilst the set from the opposite limb

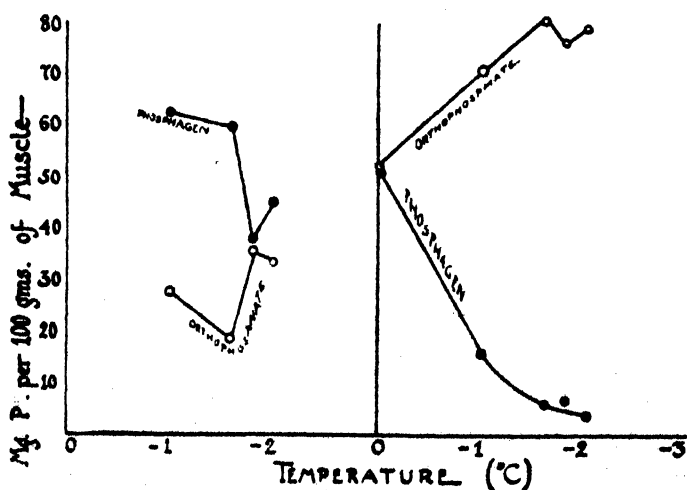


FIG. 6.—Phosphagen and inorganic phosphate content of muscles thawed in oxygen (on the left) and in nitrogen (on the right).

was thawed in nitrogen. It appears that oxygen is necessary for the resynthesis of phosphagen in these circumstances, and the conditions here are reminiscent of those necessary for the resynthesis of glycogen. In the recovery of a muscle from a short tetanus, phosphagen may be resynthesised in the absence of oxygen (Nachmannsohn, 1928). The source of energy for this resynthesis is not indicated, but it is presumably derived from the breakdown of glycogen to lactic acid. Although this reaction is occurring in the thawed muscles, the energy liberated is apparently not available for the resynthesis of phosphagen.

In the muscles thawed in oxygen, but not in those thawed in nitrogen, there is evidence of a slight increase in hexose phosphoric esters. (In nine experiments the increase was between 2 and 6 mg. of hexose phosphoric acid phosphorus.) This increase is probably a result of the increase in concentration of inorganic phosphate in the muscle. A similar increase has been observed by Eggleton (1929) in prolonged anaerobiosis.

Conclusion.—These experiments give further evidence of the exceedingly close relationship between the "circulatory system" of carbohydrate in muscle and that of phosphagen. There is, however, nothing in these results to indicate what is the individual function of each system, nor how they are functionally interrelated.

It has been pointed out in a previous paper from this laboratory (Smith and Moran, 1930), that the only change in the system which is necessary to bring about the breakdown of these complex molecules is an increase in the concentration of the plasma. It might be assumed that the effect of altering the ionic environment is to favour enzymic hydrolysis, but it is hardly likely that in the frozen state two separate enzymes would react in an identical way to this change, giving a maximum rate of hydrolysis at the same temperature, nor is it likely that the two systems are so closely bound together as to be controlled by one single enzyme. It rather appears that in the presence of an enhanced salt concentration a greater liberation of energy is required to maintain the intactness of the muscle system, and in a sense this breakdown may be regarded as an enormously increased resting metabolism. A considerable increase in resting metabolism has, in fact, been observed by Fenn (1930) in association with the non-irritable condition of muscle. Dulière and Horton (1929) attribute this condition to a disturbance of the distribution of potassium ions in the muscle, so that there is some support for the view that an increase in metabolism can be associated with a change in the ionic environment of the muscle.

In accordance with this view, it would be anticipated that death would occur when the demands of the system on the available energy became too great for the supply. This appears actually to be the case, for the temperature at which death in the muscle is instantaneous coincides with the point of maximal breakdown of glycogen and phosphagen (Smith and Moran, *loc. cit.*).

Summary.

In frozen frogs' muscles phosphagen breaks down to inorganic phosphate and creatine, the maximum rate of breakdown taking place at the same temperature (-2.6°C.) as the maximum rate of breakdown of glycogen.

Muscles frozen for 24 hours not lower than -1.5° recover their initial phosphagen content on thawing. Below -2.5° there is no recovery. Between these temperatures recovery is partial, owing to differences in susceptibility to freezing of different fibres. Oxygen is necessary to this recovery process.

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The Oxygen Uptake of Frozen and Thawed Muscles.

By E. C. SMITH, Low Temperature Research Station, Cambridge.

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In connection with the chemical changes which muscle undergoes on freezing and thawing (Smith, 1929; Smith and Moran, 1930), it appeared of interest to determine the degree of injury suffered by the respiratory mechanism of the muscle as a result of freezing. It was hoped, in particular, that some light might be thrown on the nature of the injury to the machinery involving the synthesis of glycogen from lactic acid, since in this process the energy for the building up of the greater part of the lactic acid is derived from the oxidation of a smaller fraction. A muscle frozen for 24 hours at -1.6°C . and then thawed is found to have lost this faculty of oxidative synthesis. The breakdown of the synthetic mechanism might be due to the destruction of the mechanism of oxidation, of the actual synthetic enzyme, or of the coupling mechanism by which the energy of the former reaction is made available for the process of synthesis.

The first possibility could be tested by actual experiment, and the major part of this paper gives the results of experiments made with this end in view. In addition, some measurements of the respiration of muscles actually in the frozen state have been made. As might be expected, the oxygen uptake gradually diminishes with falling temperature. There is no sign of any sudden change in the neighbourhood of the critical temperature which might be associated with the change in behaviour of the muscle with respect to the carbohydrate metabolism.

No attempt has been made to measure the carbon dioxide evolved by the frozen muscles, since the low rate of diffusion through the semi-solid mass of the tissue, and the increase in acidity as a result of the liberation of lactic acid would have introduced too great difficulties in the interpretation of such measurements.

The Oxygen Uptake of Frozen Muscles.

The oxygen uptake was measured in a Barcroft apparatus. Each chamber had a total capacity of about 10 c.c., that one containing the muscles (two frogs' sartorii, averaging 0.15 gm. muscle) having a small tap at the lower end

to admit of its being filled with oxygen. A change in volume of 0.001 c.c. corresponded to a difference of 0.2 cm. on the manometer, so that an uptake of 0.01 c.c. per gram of muscle per hour over a period of 8 hours could readily be measured.

The chambers were immersed in a bath of brine maintained by thermostatic control at the required freezing temperature in a cooled, insulated room. Measurements of oxygen uptake were made at zero and at several temperatures between zero and -4°C. , at which temperature respiration was reduced to an exceedingly low value. The results are shown in fig. 1.

The upper curve shows the amount of liquid phase remaining in the frozen

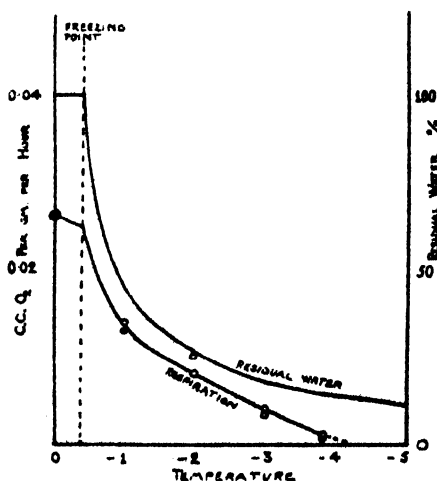


FIG. 1.—Oxygen uptake of muscles in the frozen state.

muscle at temperatures between the freezing point and -5° . It will be observed that the oxygen uptake is diminished *pari passu* with the removal of water between the freezing point and -3° , and it would seem, therefore, that the extent of oxidation in this range is determined by the actual volume of liquid remaining in the muscle. It does not seem necessary to assume that the tissue is suffering from oxygen starvation in any other sense—such, for instance, as would be brought about by hindered diffusion through a shell of ice. This factor, in all probability, does not enter in until respiration drops to zero at -4° , at which temperature there is still 10 to 15 per cent. of water in the muscle. In any case, oxygen starvation cannot in itself be the cause of the breakdown of glycogen, since, as was pointed out in an earlier paper (Smith, 1929) the rate of production of lactic acid between -2° and -4°C. is far greater than in muscles stored in nitrogen at zero.

The oxygen uptake of the muscle is not, therefore, included in the group of reactions which are accelerated by freezing. So far as is known up to the present, these reactions are limited to the breakdown of glycogen and the breakdown of phosphagen.

The Oxygen Uptake of Thawed Muscles.

Meyerhof (1919) made the broad observation that muscles which had been frozen at -3° C. had, on thawing, a normal oxygen uptake, whilst muscles which had been frozen at the temperature of solid CO_2 and ether had practically lost the faculty of respiration. The critical range of freezing has been carefully explored, and it has been found that recovery of respiration is complete in muscles frozen 24 hours at -2.4° , is partial in muscles frozen 24 hours between -2.4° and -3° , and is almost nil (but is never completely abolished) in muscles more severely frozen than this.

Fenn has recently shown (1930) that sartorius muscles of the frog are liable to give abnormally high values for oxygen uptake unless they are subjected to a short soaking in Ringer's solution after being removed from the body. The sartorius muscles used in the following experiments have, therefore, been treated in this way both on removal from the body, and after thawing, before any measurements of oxygen uptake have been made.

The muscles of two animals were used for each experiment. One muscle of each animal was kept in moist air at zero whilst the other muscles were frozen, and the oxygen uptake of the treated and of the untreated muscles was then measured under identical conditions at zero. The measurements extended over a period of 5 to 8 hours. In this time the uptake was generally steady, but over longer periods (up to 24 hours) a slight falling-off in oxygen consumption was usually observed. The uptake was calculated in cubic centimetres of oxygen per gram of muscle per 24 hours.

In all cases the period of freezing was 24 hours. The muscles on which these observations were made were, therefore, in a comparable condition to those used in earlier experiments (Smith, 1929; Kreps, 1931).

In fig. 2 the ratios of the oxygen uptake of the frozen and thawed muscles to that of the unfrozen controls are plotted against the temperature of freezing.

From these results the most important conclusion may be drawn that a muscle frozen irreversibly may yet have a considerable oxygen uptake. Judged by the criterion of irritability, muscles frozen for 24 hours at -2.4° are completely functionless, they have lost the power to synthesise glycogen and phosphagen, yet oxygen is used at a rate equal to, or greater than, the normal.

It necessarily follows that the destruction of the respiratory function of the muscle is not responsible for the loss of its contractile and synthetic faculties.

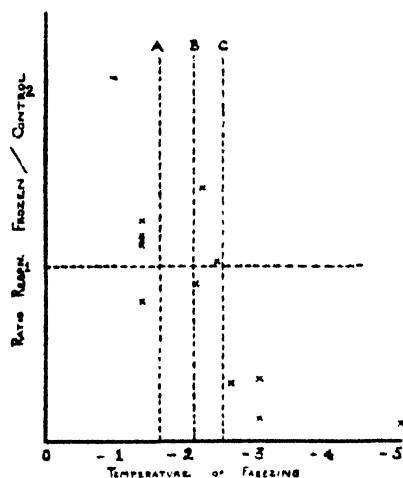


FIG. 2.—Oxygen uptake of thawed muscles compared with resting control.

In normal muscle, the energy for the synthesis of lactic acid to glycogen is derived from the oxidation of some member of the carbohydrate "circulatory system," probably lactic acid itself. It was realised that a muscle might show a normal oxygen uptake and yet have lost the faculty for oxidising lactic acid (or its equivalent in carbohydrate) which is a necessary part of its synthetic mechanism. The following experiments show that lactic acid is lost when these irreversibly frozen muscles are thawed and kept in oxygen, and that a large part of the oxygen which is absorbed must necessarily be used in the process of its combustion.

The gastrocnemius and sartorius muscles of frogs were frozen for 24 hours at a temperature between -2.1° and -2.4° . On thawing and soaking in Ringer's solution, specimen muscles were found to be non-irritable. The muscles of one side of six animals were allowed to thaw and stored for 24 hours at $+5^{\circ}$ in oxygen, those of the other side were thawed and kept at the same temperature in nitrogen rendered free from oxygen by passage through a Berkefeld candle immersed in sodium hydrosulphite solution. Lactic acid was then determined in each batch. The figures obtained are given in Table I.

The muscles stored in nitrogen are seen to have, on the average, 0.06 per cent. of lactic acid in excess of those stored in oxygen. The experiments were carried out during the months of September and October, and it so happened that the glycogen content of the muscles was remarkably constant, and almost

Table I.—Disappearance of Lactic Acid from Thawed Muscles stored in Oxygen.

Experiment.	Muscle.	Lactic acid percentage.		Lactic acid oxidised percentage of that present.
		In nitrogen.	In oxygen.	
1	Gastrocnemius	0.73	0.66	9.5
2	"	0.72	0.67	7.0
3	"	0.69	0.66	4.5
4	Sartorius	0.85	0.76	10.5
5	"	0.80	0.75	6.0

sufficient in amount to give a true rigor maximum. In five determinations made to test the constancy of the level reached in rigor, the mean initial glycogen content was found to be 0.67 per cent. (equivalent to 0.72 per cent. of lactic acid) and the actual level reached by storage in nitrogen saturated with chloroform vapour was 0.72 per cent. of lactic acid. Any removal of lactic acid was readily apparent in such muscles, whereas muscles with higher glycogen content than that necessary to provide the rigor maximum of lactic acid (0.80 per cent. in gastrocnemius and 0.85 per cent. in sartorius) would have been able to restore from this excess glycogen the lactic acid removed by oxidation, and so mask its disappearance. The muscles of animals examined one month later were found to have considerably higher and more variable glycogen contents (up to 1.8 per cent.) and for this reason could not have served as material for these experiments.

The oxygen equivalent to the lactic acid removed is of very much the same order as the oxygen which was found to be utilised in this period. Assuming complete combustion to carbon dioxide and water, 0.06 gm. of lactic acid would require 0.064 gm. of oxygen. This corresponds to an uptake of 0.44 c.c. per gram of muscle per 24 hours. The actual utilisation of oxygen by such muscles is something less than 0.6 c.c. per gram per 24 hours, so that at least 75 per cent. of this oxygen uptake can be accounted for by the oxidation of lactic acid.

The irreversibly frozen muscle has not necessarily, therefore, lost the capacity of oxidising lactic acid. It has, however, lost the power to synthesise glycogen, consequently the part of the mechanism which is destroyed by freezing is the actual synthetic machinery of the muscle, and not that part which supplies energy for the working of the machine. It is quite conceivable that the same considerations apply in the case of phosphagen. In intact muscle phosphagen

is in part resynthesised anaerobically after it has been broken down in the course of muscular contraction. Kreps (1931, *supra*) was unable to demonstrate any resynthesis of phosphagen in irreversibly frozen muscles although ample energy is liberated in the thawed muscle by the breaking down of glycogen to lactic acid.

Meyerhof (1930, p. 282) stresses the fact that the coupling of the energy-producing reaction with the energy-absorbing reaction in the synthetic process is inseparably connected with the structural elements of the muscle. It would appear then that the changes brought about in the first place are due to alteration of those structural elements whose particular function is the maintenance of the carbohydrate "circulatory system" of the muscle. Damage to this system is followed by a diminished power of contraction and damage to the mechanism of synthesis of phosphagen. If it be assumed that the gradual loss of function in the two latter instances is due to differences in susceptibility to freezing of different fibres, the data at present available may be expressed as follows (see also fig. 2) :—

Function.	Temperature at which function is lost in 24 hours in—	
	Most susceptible fibres.	Least susceptible fibres.
Synthesis of glycogen	-1.6	-1.6 A
Contractility	-1.6	-2.1 B
Synthesis of phosphagen	-1.6-1.8	-2.6 C
Respiration	-2.4	-3.0

The several functions of synthesis and contractility appear to possess no really close connection, since the temperatures at which complete loss of function is observed differ so widely in the three instances. It cannot be said that loss of contractility is dependent on the failure of either the glycogen system or the phosphagen system, for the muscle retains contractility after much more severe treatment than that required to destroy the glycogen system, but contractility is lost whilst some 50 per cent. of the fibres are capable of synthesising phosphagen. This would point to there being separate sites in the muscle structure with which these three functions are associated.

The oxygen uptake of frogs' muscles in the frozen state diminishes to zero at -4°C .

Muscles frozen 24 hours at a temperature not lower than -2.4° recover their initial oxygen uptake on thawing. Below -3° the oxygen uptake on thawing is reduced to less than 10 per cent. of the resting uptake.

The loss of contractility and of the capacity to synthesise glycogen and phosphagen observed in thawed muscles is not due to destruction of the respiratory faculty of the muscle.

The measurements recorded in this paper were carried out by Mr. H. F. Davis.

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The Molecular Statistics of an Enzyme Action.

By J. B. S. HALDANE.

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Until recently no exact figures were available concerning the rate of transformation of the enzyme-substrate compound. For example, while Willstätter and Pollinger's (1923) best peroxidase preparation could activate over 2000 times its weight of H_2O_2 per second under favourable conditions, neither its degree of purity nor its molecular weight is known. Hence such calculations as that of Haldane (1930, p. 176) based on this work are necessarily very rough. Zeile and Hellström (1930) have found that horse-liver catalase is an iron-porphyrin compound with a spectrum resembling that of alkaline hæmatin, and that its amount, reckoned in milligrams of combined iron, can be measured photometrically with great accuracy by converting it into pyridine-hæmochromogen. Thus, although it has not been obtained pure, one can now estimate the activity of a molecule of catalase. In what follows

the assumption is made that each molecule of catalase contains only one atom of iron. If this is not so, as in the case of hæmoglobin, where the molecule contains four iron atoms, the calculations given below are true, not for the molecule as a whole, but for each of the active centres on it, since the iron-porphyrin complex is clearly the active centre, as shown by the effects of cyanide.

Zeile and Hellström worked at 0° C. and the optimal p_H of 6.6, with 0.01 M. H_2O_2 , which disappears according to the unimolecular law, and express their results as unimolecular velocity constants per milligram Fe per litre. Their unit of time is a minute. Moreover, an examination of Zeile's (1930) protocols shows that he employs decimal instead of natural logarithms. Hence to reduce the published velocity constants to the normal unit, they must be multiplied by $2.3026/60$, or 0.03838. For horse-liver catalase solutions containing x mg. Fe per litre, Zeile and Hellström found a unimolecular velocity constant of $2513x$, the standard error being only $86x$; or neglecting one rather dubious figure, the velocity constant is $2531 \pm 63x$. Zeile (1931) found a rate of about $8000x$ for a catalase prepared from seedlings, but the error is considerably greater. In ordinary units the constant* for liver catalase is $97.14x$, i.e., 1 mg. Fe destroys 0.9714 gram-molecule of H_2O_2 per second, in 0.01 M. solution, and 1 gram-molecule of enzyme destroys 5.42×10^4 gram-molecules of H_2O_2 per second. In other words a molecule of enzyme destroys 5.42×10^4 substrate molecules per second at 0° C. and 10^{-2} M. substrate concentration. Under the same conditions plant catalase destroys about 1.7×10^5 substrate molecules per molecule per second.

Euler and Josephson (1927) found that at 0° C. the velocity of H_2O_2 destruction by liver catalase could be represented by the equation $v = Vx/(x + K)$, where $K = 0.025$. Hence the maximum velocity V , which is nearly reached in 0.2 M. H_2O_2 , is $[1 + (K/x)]v$. Substituting the values of x and K we find that in a strong peroxide solution where the velocity is independent of substrate concentration, one enzyme molecule destroys 1.90×10^5 substrate molecules per second at 0° C.

The effect of temperature on the reaction velocity has been rather inadequately studied. The enzyme is rapidly destroyed by H_2O_2 at temperatures above 0° C., and although this can be allowed for, it brings in an element of uncertainty. The only satisfactory data for liver catalase are those of Williams (1928) which only cover the range from 10° to 20°. However, Nosaka (1928) had studied

* i.e., the value of k in the equation $ds/dt = ks$, where s is the substrate concentration, and t is measured in seconds.

blood catalase from 0° to 50° . This behaves fairly like liver catalase where the data are comparable, and may be the same substance. Nosaka found that the velocity at 40° C. was 2.91 times that at 0° C. Thus at 40° C. a catalase molecule can destroy about 5.5×10^5 H_2O_2 molecules per second.

As the reaction has a temperature coefficient, only a small proportion of the enzyme molecules are active at a given moment, even when the substrate concentration is so high that almost all of them are combined with substrate. This is partly no doubt due to the fact that even at the optimum p_{H} not all the enzyme molecules have the appropriate charge. But the flatness of the optimum shows that this correction is not very important for catalase, and the effect of temperature on ionisation probably does not have a large effect on the apparent critical increment. Williams found an apparent critical increment Q of 3080 calories per gram-molecule at 15° – 20° C., and 4380 from 10° – 15° C., in strong peroxide solutions at p_{H} 6.8. Both of these values are uncertain, and the difference between them still more so. At best they can only give us the order of magnitude of the fraction of molecules activated.

On Arrhenius' theory the proportion of molecules which are active at any given instant is $e^{-Q/RT}$. If we take $Q = 3730$ at 15° this works out at $1/646$. So if the reaction velocity at 15° C. is 1.431 times that at 0° , the rate of transition of an active molecule is $646 \times 1.431 \times 1.90 \times 10^5$, or 1.80×10^8 per second, and the mean life of an active catalase-hydrogen peroxide molecule is 5.6×10^{-9} second.

Other more modern hypotheses give figures of the same order but larger. Moelwyn-Hughes (1929) has applied to enzyme actions Fowler and Rideal's (1927) and Hinshelwood's (1926) equation, which may be written

$$\frac{k}{B} = \frac{e^{-x} x^f}{f!},$$

where k/B is the fraction of molecules which are active, $x = E/RT$, E being the critical increment, and $f = \frac{1}{2}S - 1$, where S is the number of square terms in the molecule's energy function which are concerned in activation. f is, on certain hypotheses, equal to one less than the number of degrees of freedom. If then Q be the apparent energy of activation, $E = Q + fRT$. Hence from Williams' data on the apparent critical increment, and its decrease with temperature, we find $f = 131$, $E = 77,000$. These values are very uncertain, but agree well with Moelwyn-Hughes' figures of $f = 121$, $E = 84,000$ for the hydrolysis of starch by malt amylase, for which much more reliable data exist. In any case the precise value of f makes little difference to our final result.

With the above figures $Q = 5680$ at 0°C. and $B/K = 42.9$, whence the mean life of an active molecule is 1.24×10^{-7} second.*

A figure of this order is more plausible than the lower one, because on the modern theory of chemical reactions we expect a reaction with a large value of B , i.e., a short-lived active molecule, to have a high critical increment, and this involves a large number of degrees of freedom being concerned in the reaction, if the apparent critical increment be small. The mean life of 10^{-7} second is unique in being derived from purely chemical data, and does not involve any hypothesis as to collisions. All the comparable figures known to me are derived from spectroscopic data, and refer to the mean "lives" of activated atoms or molecules which are ended by radiation. These "lives" range from 6×10^{-9} second for Ca^+ atoms in the P state (Milne), to 2.1×10^{-5} second for a transformation of the Cs atom (Tolman). These figures are taken from Dushman's article in Taylor's "Treatise on Physical Chemistry" (1931). The agreement is rather striking, considering that catalase is a colloid.

One chemical action is available with which the break-down of catalase— H_2O_2 may be compared, namely the break-down of oxyhæmoglobin, studied by Hartridge and Roughton (1923). The molecules concerned are similar, though the porphyrin-bound iron appears to be bivalent in hæmoglobin, but trivalent in catalase. They found a unimolecular velocity constant of about 70 in moderately acid solutions at 15°C. , the corresponding figure for catalase-hydrogen peroxide being about 2.7×10^5 . On the other hand they found the surprisingly large Q_{10} of 3.8, corresponding to $Q = 22,000$. So $Q/RT = 38.5$. Hence the proportion of excited molecules on Arrhenius' theory would be $e^{-38.5}$, and the mean life of an excited molecule 3×10^{-10} second, an impossible figure. However, Hartridge and Roughton's data only extend over 20°C. , and could be fitted to Fowler and Rideal's formula about as well as to Arrhenius'. Taking $f = 131$, a reasonable assumption in view of the similarity of hæmoglobin and catalase, $E = 65,000$, and $B/K = 3243$, giving a mean life of 4.0×10^{-6} second for the excited oxyhæmoglobin molecule, a value of the expected order. The number of degrees of freedom concerned is therefore possibly of the order of 131, and probably at least 50.

At about 80° – 90°C. , on this view, oxyhæmoglobin would dissociate about as rapidly as catalase-hydrogen peroxide, with a low temperature coefficient. The hydrolytic enzymes (like catalase) all have low temperature coefficients, the apparent critical increments falling rapidly with temperature, and low affinities

* This figure refers only to the break-down into catalase, H_2O , and O . As some molecules break-down into catalase and peroxide, the mean life, allowing for this, is shorter.

for their substrates. This is intelligible if the values of E and f are both high, a natural assumption in the case of such complex molecules. But their intense reactivity probably depends on the fact that f is so large that an appreciable fraction of all the enzyme-substrate compound molecules is active. On the other hand, many oxidising enzymes have an equally low temperature coefficient, which does not fall off greatly with temperature, as pointed out by Haldane (1930, p. 65). Here f must be relatively small. But they generally have a high affinity for their substrate, and this may be associated with a high heat of reaction, whereas in the case of the only hydrolytic enzyme examined, the corresponding heat is low. This heat of reaction may take the place of the extra degrees of freedom in activating the substrate.

We must now consider the behaviour of catalase from the standpoint of collision. There are much more than enough collisions with H_2O_2 to keep up the supply of active molecules. With regard to peroxide we have three reactions, writing E for the enzyme molecule :—

1. $E + H_2O_2 \rightarrow E \cdot H_2O_2$, velocity $k_1(e - p)x$,
2. $E \cdot H_2O_2 \rightarrow E + H_2O_2$, velocity k_2p ,
3. $E \cdot H_2O_2 \rightarrow E + H_2O_2 + O$, velocity k_3p ,

where x is the concentration of H_2O_2 , e that of total catalase, p that of combined catalase. Then, as pointed out by Briggs and Haldane (1923),

$$-\frac{dx}{dt} = \frac{k_3ex}{x + \frac{k_2 + k_3}{k_1}}.$$

At $0^\circ C$. $k_3 = 1.9 \times 10^5$, and $(k_2 + k_3)/k_1 = K = 0.025$ M. We can at once obtain a minimum value for k_1 , and hence for the number of effective collisions per second. In 0.01 M. solution at $0^\circ C$., 5.42×10^4 H_2O_2 molecules are destroyed per enzyme molecule per second. But since $K = 0.025$, $x = 0.01$, $x/(x + k)$ or $2/7$ of the enzyme molecules are combined, so $2/7$ of all collisions between peroxide and catalase find the catalase molecule occupied. Thus the number of collisions between H_2O_2 and unoccupied catalase which lead to reaction is $1.4 \times 5.42 \times 10^4$, or 7.6×10^4 . But the probability that an enzyme-substrate molecule should react to give off oxygen is $k_3/(k_2 + k_3)$, so the actual number of collisions leading to combination is $7.6 \times 10^4 [1 + (k_2/k_3)]$, and $k_1 = 7.6 \times 10^6 [1 + (k_2/k_3)]$.

We have no data for the estimation of k_2/k_3 . Haldane (1930, p. 81) showed that in the case of prunase it must be large. But in that of catalase if k_2 were much larger than k_3 , say over 100 times, we should be led to a value

of less than 10^{-9} second for the mean life of an enzyme-peroxide molecule, which is improbably small. Hence k_2/k_3 may be small, and probably does not exceed 100.

In dilute solutions $-\frac{dx}{dt} = \frac{k_1 k_3 ex}{k_2 + k_3}$, in strong solutions it equals $k_3 e$. As

Williams found that the temperature coefficient was nearly independent of the peroxide concentration, this means that $k_1 k_3 / (k_2 + k_3)$ has the same temperature coefficient as k_3 , i.e., that $k_1 / (k_2 + k_3)$ is independent of temperature. This can be most easily explained if k_2 is somewhat larger than k_3 , and has the same temperature coefficient as k_1 .

We can compare the union of catalase and peroxide with that of hæmoglobin and oxygen, studied by Hartridge and Roughton (1925). They give the constant k_1 of the reaction $\text{Hb} + \text{O}_2 = \text{HbO}_2$ as 2070, 3145 and 6150, for three different blood samples. As, however, they reckoned concentrations in millimols per litre, the values of the velocity constant in ordinary units would lie between 2×10^6 and $6 \cdot 15 \times 10^6$. This is clearly of the same order as the value somewhat exceeding $7 \cdot 6 \times 10^6$ found for catalase. Putting the matter in another way, 2 to 6×10^4 molecules of O_2 unite with one of reduced hæmoglobin per second in 0.01 M. O_2 solution, as compared with $> 7 \cdot 6 \times 10^4$ for catalase. The true value for catalase is perhaps somewhat larger than 10^5 , but considering the great difference between hæmoglobins even from animals of the same species, we should not expect complete agreement. Hartridge and Roughton found that the rate of combination was independent of temperature, which presumably means that every O_2 molecule striking the hæmoglobin molecule on a certain small area with a certain orientation unites with it, regardless of its energy. However the slower reaction $\text{Hb} + \text{CO} \rightarrow \text{Hb CO}$ has a Q_{10} of about 2.

In view of the similarity between O_2 and H_2O_2 on the one hand, catalase and hæmoglobin on the other, we should expect the geometrical conditions for combination to be much the same in the two cases. Hence if k_1 for catalase had even a moderate temperature coefficient, i.e., only a few of the collisions satisfying a certain geometrical condition led to combination, we should expect it to be smaller than the same coefficient for hæmoglobin, whereas it is larger. Hence k_1 has probably at most a small temperature coefficient, and possibly none. If so k_2 must exceed k_3 , but be independent of temperature. In this case the heat of reaction of catalase and hydrogen peroxide, like that of saccharase and sucrose (Euler and Laurin (1920)) is very small.

Any attempt to apply the kinetic theory is inevitably speculative. The

number of collisions in a liquid is equal to the number calculated from gas theory multiplied by a factor which is probably greater than unity, but whose value is very uncertain. Jowett (1929) estimates it at 10 to 14 in aqueous solutions, and ascribes to it a negative temperature coefficient, which, even allowing for the $T^{\frac{1}{2}}$ term in the collision rate, gives a Q_{10} of about 0.9 for the collision rate at ordinary temperatures. We can obtain a rough idea of the efficiency of collisions as follows. We calculate the number of times that a molecule of H_2O_2 or O_2 in 0.01 M. solution collides with a given point on the enzyme surface, *i.e.*, in which the centre of the colliding molecule strikes the enzyme within a circle of radius equal to its own. The diameter of an oxygen molecule is about 2.71×10^{-8} cm., according to Sutherland (1910), so this area is 5.77×10^{-16} cm.². The root mean square velocity $\bar{u}$ of O_2 at 0° C. is 4.61×10^4 cm./sec. Hence the fraction of a gram-molecule striking this area per second in a 0.01 M. solution, as calculated from the gas laws, would be $10^{-5} \times 3/13 \times 4.61 \times 10^4 \times 5.77 \times 10^{-16}$, or 5.9×10^{-17} . This is equivalent to 3.6×10^7 molecules, corresponding to $k_1 = 3.6 \times 10^9$. The figure is almost the same for H_2O_2 . The velocity of the enzyme or hæmoglobin can be neglected, as it is a colloid. (The figure 10^{-5} in the above product is the fraction of a gram-molecule per cubic centimetre.) In a liquid the corresponding figure in 0.01 M. solution perhaps lies between 10^8 and 10^9 , whereas the number of effective collisions lies between 10^4 and 10^5 per second. Hence the geometrical conditions for an effective collision must be rather stringent. This is perhaps due to the fact that the Fe atoms in hæmoglobin and catalase are each co-ordinated with four heterocyclic ring N atoms. If, however, oxygenase is a similar compound this cannot be the main reason, since, as will be seen later, oxygenase has a k_1 much larger than the above figure.

Hennichs (1926) found that 1 gm. of liver decomposed H_2O_2 at a rate given by $k = 20$. Hence it must have contained 0.008 mg. Fe in catalase. This is a very large amount, but it must be remembered that catalase has a low apparent affinity for its substrate, and as the H_2O_2 concentration in living cells is certainly small, must work at 0.1 per cent. or even less of its maximum possible velocity. Most enzymes are probably much more nearly saturated with their substrate.

It is important to realise that the figures obtained above for catalase are not by any means typical for enzymes, partly because the temperature coefficient of catalase action is very low, *i.e.*, an unusually large proportion of its molecules are active at any moment. For example, Warburg and Kubowitz (1928), measuring the CO uptake of a yeast suspension, found a con-

centration of $< 4 \times 10^{-7}$ gm. Fe combined in *Atmungsferment* (oxygenase) per gram dry weight, i.e., $< 7 \times 10^{-9}$ gram-molecule of oxygenase. As this yeast had an oxygen uptake per gram dry weight of 66 c.cm. O_2 per hour, 1 gram-molecule of oxygenase activates > 115 gram-molecules of oxygen per second, i.e., $k_3 > 115$. This figure resembles that for hæmoglobin, and the reaction concerned probably has a Q_{10} of slightly over 2. Now K , the apparent dissociation constant of the oxygenase-oxygen compound, was found by Warburg (1927) to be $< 5 \times 10^{-8}$. Therefore $(k_2 + k_3)/k_1 < 5 \times 10^{-8}$. So $k_1 > 2 \times 10^7 \times 115 = 2.3 \times 10^9$.

If, as Warburg and Kubowitz (1929) found in the case of *Micrococcus candidans*, $(k_2 + k_3)/k_1 < 10^{-8}$, we should have $k_1 > 10^{10}$. If we allowed a hit of the oxygen molecule over an area 3 or 4 times as large as considered above, such a value would be just possible on the gas theory. But if, as seems probable, oxygenase is an iron-porphyrin compound, the iron atom is probably well screened. In this case the collision number in liquids must be substantially greater than that in gases. The study of enzymes certainly offers us the possibility of investigating this question. Unpublished work of my colleague Mr. Mann on peroxidase suggests that the collisions in liquids are several hundred times more frequent than they would be if the gas laws held good.

Yeast saccharase was found by Euler and Ericsson (1922) to have a molecular weight of about 1.4 times that of egg albumin, i.e., about 50,000. Its apparent critical increment is 38,400 — 50 RT according to the data of Euler and Laurin. Thus at 15° C., according to the Fowler-Rideal-Hinshelwood formula, one molecule in 146 is active. One gram of purified saccharase at this temperature can hydrolyse 6 grams of sucrose per second, according to Willstätter, Schneider and Wentzel (1926). Hence if $1/c$ be the purity of their preparation, 1 molecule of saccharase can hydrolyse about 900 c sucrose molecules per second, i.e., $k_3 = 900 c$, and since $(k_2 + k_3)/k_1 = 0.026$ on an average, $k_1 > 3.5 \times 10^4 c$. In this case k_2 is much larger than k_3 , so k_1 is probably of the order of 10^6 at least. The mean life of an active molecule is $< 1/(146 \times 900c)$, or $7.6/c \times 10^{-6}$ second. These values are, of course, only approximate, but it is satisfactory to find a mean life and collision rate of the same order as for catalase. The life would be longer if there were several active centres per enzyme molecule.

Although enzymes in the cell are in general acting neither at their optimal temperature, p_H , nor substrate concentration, it would seem from the above examples that an enzyme molecule must commonly transform 100

or more substrate molecules per second. As a very active cell, such as baker's yeast, metabolises about 10^{-6} of a gram-molecule of O_2 per gram dry weight per second, we can get some idea of the possible complexity of the transformation undergone in it. If 5 per cent. of the dry weight of the cell consists of enzymes of an average molecular weight of 50,000, then 1 gram dry weight contains 10^{-6} gram-molecule of such enzymes, and less than 1 per cent. of this would be required for any particular process, *e.g.*, the activation of O_2 . In other words the average atom, on its metabolic path through the cell, may be dealt with by more than 100 catalysts in succession.

Summary.

Zeile and Hellström's data show that a molecule of catalase at $0^\circ C.$ catalyses the destruction of about 2×10^6 H_2O_2 molecules per second. The mean life of an active catalase-hydrogen peroxide molecule is about 10^{-7} second. The velocity constant for the union of catalase and hydrogen peroxide exceeds 7×10^6 . These values are compared with similar data relating to other enzymes and to hæmoglobin.

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*The Chemistry of Irradiated Ergosterol.**

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At the invitation of the Secretary of the Royal Society, I present to you an account of my work on the chemistry of irradiated ergosterol.

Since it became known that ergosterol, on irradiation with ultra-violet rays, is converted into an antirachitic substance of high activity, presumably identical with the vitamin D of cod-liver oil, the study of the most favourable conditions for the course of this photochemical process has continued, together with attempts to prepare the active substance in pure crystalline condition, and to obtain knowledge of its physical and chemical properties.

But, so long as success in this purification had not been attained, the investigations had to be carried out with the crude irradiation product, the vitamin content of which was unknown; and in order to evaluate the results satisfactorily, use had to be made almost always of protracted physiological experiments. The information which it was possible to obtain in this way, concerning the nature and behaviour of the vitamin, may first be summarised, attention being called to those points which could not be elucidated without isolating the pure vitamin in crystalline form.

Irradiation.

May I make, in the first place, some remarks on the process of irradiation. In this process, no state of equilibrium is attained between ergosterol and its irradiation products, such as is reached between trans- and cis-cinnamic acid, for example. If the irradiation is continued for a sufficiently long time, the ergosterol disappears completely; but the highly active antirachitic material (the vitamin D), first formed, is only an intermediate product of the chemical transformation of ergosterol, and is itself sensitive to irradiation with ultra-violet rays; it is gradually converted into inactive, over-irradiated products, of which two have been obtained in crystalline form. These two products of over-irradiation are end-products of the chemical transformation, and neither

* This communication was presented by Professor Windaus as a contribution to the discussion on "Recent advances in the chemistry of the vitamins," held at the Royal Society on June 18, 1931.

can be converted into the other; both must, therefore, be independently formed, and not the one from the other. Hence it follows, that in some phase of the reaction a separation into two photochemical series must occur. This fact is important. Thus, Reerink and van Wijk have found that, when ergosterol is activated with long-wave light and the unchanged ergosterol is removed, the reaction product always has exactly the same absorption-spectrum, no matter whether 10, 20, 30, 40 or 50 per cent., but not more, of the ergosterol has been transformed. The Dutch workers consequently conclude, that the substance possessing this spectrum is the pure vitamin D. It appears equally possible, however, that during the ultra-violet irradiation of ergosterol several substances are formed simultaneously, always in the same proportion, and that the spectrum measured by Reerink and van Wijk is that of the mixture, having a constant composition.

Physical Properties.

Turning now to the active irradiation product itself: in its physical properties it differs widely from ergosterol; yet, until the pure, crystalline vitamin D has been isolated, it is especially difficult to deduce the physical properties of the vitamin from those of the amorphous irradiation product. It is striking to observe, how much lower the melting point, and how much greater the solubility, of the crude irradiation product are than those of ergosterol.

The change in *optical rotation*, which occurs during irradiation, is characteristic. Ergosterol, which has a strong *laevo*-rotation, is converted into an irradiation product which is feebly *dextro*-rotatory. Moreover, the rotation is very dependent on the wave-length of the ultra-violet rays used in the irradiation, being the more positive the longer the wave-length used.

As far as the *ultra-violet spectrum* is concerned, the following is the state of affairs. During irradiation the absorption spectrum of ergosterol undergoes a series of complicated changes, and finally the portion above $240\text{ m}\mu$ is almost completely eliminated, and, in parallel with this elimination, the physiological action disappears. According to the type and duration of the irradiation, characteristic bands at $248\text{ m}\mu$, at $280\text{ m}\mu$ and at $265\text{--}270\text{ m}\mu$, appear at intermediate stages.

Whereas it was originally supposed that the characteristic absorption maximum for the vitamin lay at $248\text{ m}\mu$ or at $280\text{ m}\mu$, later investigations have made it very probable that the chief absorption band of the vitamin is at $265\text{--}270\text{ m}\mu$.

But complete certainty, on this point also, can only be obtained when the vitamin is prepared in pure state.

Chemical Properties.

(1) *Behaviour to Digitonin*.—Turning now to the chemical properties of the vitamin D, it is characteristic that it does not show the typical reaction of natural sterols, namely formation of a sparingly soluble addition compound with *digitonin*. This behaviour makes possible a quantitative separation of the unattacked ergosterol from the products of irradiation, and is therefore of practical importance. The significance of this result, however, should be accepted with reserve. For, although it is true that sterols and sterol-derivatives only give a precipitate with digitonin if their hydroxyl group is intact, yet it is not permissible to maintain the converse, and to claim that, if a sterol-derivative is not precipitated by digitonin, a change in its hydroxyl group must have occurred.

(2) *Hydroxyl Group*.—On the contrary, I think that I have evidence that the vitamin still contains the same hydroxyl group as ergosterol itself. I need not rely on the fact that, in the crude irradiation product, just as in ergosterol itself, one active hydrogen atom can be detected by the method of Zerewitinoff, or that the products of over-irradiation of ergosterol are still monohydric, secondary alcohols. To this type of proof objections can be raised, which I need not mention here. But the presence of the hydroxyl group in the vitamin seems to me to be proved beyond question by the following experiment. If the highly active crude irradiation product is carefully treated with phenyl-isocyanate, a derivative completely lacking in antirachitic action is formed; but if this derivative is warmed with alcoholic potash, it is reconverted into a highly active material.* In my opinion, the only conclusion that can be drawn from this experiment is, that the vitamin, being a hydroxy-compound, combines with phenyl-isocyanate to form a physiologically inactive phenyl-urethane, and that this can be reconverted by hydrolysis into the original vitamin.

For the assumption that the vitamin contains a keto- or enol-group, there seems to me to be as little ground as there is for the view that the vitamin is an *epi*-derivative of ergosterol, bearing the same relation to it as borneol does to iso-borneol.

* Alternatively, the ergosterol-phenyl-urethane may be irradiated with ultra-violet light; in this way the substance is indeed changed, but acquires no antirachitic activity. But, if the irradiation product is hydrolysed, it becomes highly antirachitic.

(3) *Formula*.—What, then, is the difference between ergosterol and the vitamin ?

It can be shown that the crude irradiation product, freed from ergosterol, has the same molecular weight as ergosterol, and also that the active antirachitic irradiation product—thrice frozen out from acetone at -80° —gives on analysis figures which agree with the formula $C_{27}H_{42}O$.

It is therefore probable, but, since the vitamin content of the irradiation product is unknown, not absolutely certain, that the vitamin is an *isomeride* of ergosterol.

(4) *Double Bonds*.—Moreover, like ergosterol itself the vitamin contains several unsaturated linkages.

Towards sodium and ethylalcohol the vitamin behaves in the same way as does ergosterol. We have proved that the crude irradiation product loses its antirachitic action when it is treated with sodium and alcohol ; as in the case of ergosterol a dihydro-derivative is formed. We have, however, no definite evidence that the beautifully crystalline dihydro-derivative, which we have isolated from the reaction mixture, is actually derived from the vitamin, and not from another irradiation product.

With Diels' reagent for the detection of conjugated double bonds, maleic or citraconic anhydride, vitamin D combines only slowly at room temperature ; at 80° , on the other hand, combination is rather rapid ; the course of the reaction can be followed physiologically, since the addition compound exhibits no antirachitic activity. In view of these facts, there can be no doubt that vitamin D contains a system of conjugated double bonds, or that, in this respect, it is very similar to ergosterol.

Further we have found that the crude irradiation product, on titration with perbenzoic acid, consumes, like ergosterol itself, three atoms of oxygen ; and also, that the crystallised over-irradiation products are trebly unsaturated mono-hydric alcohols of the formula $C_{27}H_{42}O$. Therefore it is very probable, although on the evidence just given not absolutely certain, that the vitamin contains three double bonds, as does ergosterol itself.

(5) *Stability*.—The question whether vitamin D can be preserved unchanged in the absence of air, and at low temperatures, has been much discussed. Several investigators have agreed in stating that solutions of vitamin D in oil have been kept with unchanged activity for many months. On the other hand, Auhagen and myself have proved that, as far as the crude irradiation products are concerned, changes take place in their spectrum and rotation, even when air is very carefully excludud. But, with this rapid change in

spectrum and absorption, there is no corresponding decrease in physiological activity; and hence we have concluded, that the substance which changes thus with age is not the vitamin.

On the other hand, the vitamin is much more sensitive to the action of high temperature than is ergosterol. Whereas the latter can be heated *in vacuo* at 180° without undergoing real decomposition, irradiation products lose their antirachitic activity, when heated for 4 hours at this temperature. During this change a new characteristic band, between 280–290 mμ, appears in the spectrum of the crude irradiation product. It seems to me very probable, although it is not strictly proved, that this band is due to the decomposition of vitamin D.

Towards oxygen also the vitamin is less sensitive than was at first supposed. Although the crude irradiation product, when shaken for a few hours with oxygen, absorbs one mol of the gas, the antirachitic action is hardly altered in this time, and it requires several days' shaking with oxygen to destroy most of the vitamin D, even in amounts of a few grams. Consequently, it seems that the substance in the irradiation product, which is readily attacked by oxygen, is not the vitamin itself.

Toxicity.

The fact that the therapeutically valuable irradiation product can also exert a toxic action in very large doses, and that this toxicity shows itself in the experimental animals not only in loss of weight, but also, and in particular, in hyper-calcification in various organs, has excited special interest, above all in Germany. Since the crude irradiation product is doubtless a mixture, the question has been discussed as to whether the antirachitic and the toxic actions of the irradiation mixture are caused by the same, or by two different substances. The fact that, in irradiated preparations, the antirachitic and the toxic actions run parallel to each other, supports the view that both are caused by the same substance. Yet observations have also been made, which seem to indicate that the antirachitic and toxic substances are different.

Crystallised Vitamin.

In spite of many experiments on the crude irradiation product, we have not been able to solve this question concerning the source of its toxicity, and have been, consequently, continuously spurred on to attempts to obtain the vitamin itself in the pure, crystalline state. Such attempts did not seem to us com-

pletely hopeless ; for in vacuum tubes, in which we had sealed active irradiation products, crystals had repeatedly separated. Our attempts to purify these small amounts, however, had failed. We then attempted systematically, by physical and chemical methods, to separate our irradiation products into individual constituents.

The physical methods of separation, which we tried, were fractional precipitation of the irradiation products from 10 per cent. acetone solutions at -80° , and distribution of the irradiated product between petrol and methylalcohol. Both methods failed. For the chemical separation we made use of the different reactivity of the irradiation products to maleic or citraconic anhydride. Irradiation products free from ergosterol, and containing small amounts of over-irradiation products or none at all, were allowed to react at room temperature in ethereal solution with maleic anhydride; then, after 3 days, the portions which had reacted with the anhydride were removed with dilute potash, and the remaining solution was investigated. The result was that, when the portion which readily combined with maleic anhydride was removed, a product (60 per cent.) remained, which crystallised when the solvent was evaporated, and could easily be recrystallised from various solvents. The yield of crystallised material is good ; it amounted to over 50 per cent., calculated on the portion which had not reacted with maleic anhydride.

The crystals are long well-formed needles. Their specific rotation for $[\alpha]_D^{18} = +138^{\circ}$ (0.8 per cent. solution in acetone), and $+169^{\circ}$ for $[\alpha]_{5461}^{18}$. They melt sharply at $122-123^{\circ}$. Their spectrum shows a band of main absorption at 265-270 $m\mu$, the absorption coefficient of a 0.02 per cent. ethereal solution being 1.44.

Analyses and determinations of molecular weight show that the new product is certainly an isomeric of ergosterol.

The limiting antirachitic dose has been found at 0.03 γ ; thus the product is twice as active as the English standard preparation.*

It is noteworthy that the properties which we would attribute to vitamin D, from the investigation of the crude irradiation product, are reproduced almost completely by the crystalline material. Only the high positive rotation is unexpected. How the material behaves on heating at 180° , or on hydro-

* The preparation referred to is the solution of the crude irradiation product of ergosterol, issued by the Medical Research Council ; and comparison is made with the activity corresponding to the weight of ergosterol used in its preparation, 0.03 γ of the crystalline substance being found equivalent to the amount of standard solution made from 0.06 γ of ergosterol.

generation with sodium and alcohol, has not yet been thoroughly tested. Also we cannot yet make any definite statement about its toxicity; these questions, however, will be answered in a few weeks.

I shall add a few words about the relation of our work to that of the Dutch and English workers. From ergosterol, by irradiation with long-wave rays, Reerink and van Wijk (1929) have prepared an oil, which deposits crystals on cooling; these crystals, however, are said to melt already under 0° . The oil has an ultra-violet spectrum with a maximum at $265\text{ m}\mu$, and in the opinion of Reerink and van Wijk is the pure vitamin. The product isolated by these two workers is certainly different from ours; apart from the melting point, preparations which we have made according to the directions of the authors have a specific rotation for $[\alpha]_D$ about $+30^{\circ}$, whilst our crystallised substance has $[\alpha] = 138^{\circ}$. Moreover, there are certain differences in the short-wave part of the spectrum.

I believe, that the irradiation product of Reerink and van Wijk contains, indeed, a high percentage of our crystalline substance, but that still other products of photochemical action, for example, that having the absorption band at $248\text{ m}\mu$, are also present.

The English work to which I will refer is that of Bourdillon (1930) and his co-workers. By high-vacuum distillation of the crude irradiation product these workers obtained in poor yield (some milligrammes) crystals, which could be recrystallised from dilute alcohol; the best sample of 1.5 mg. melted at $113\text{--}115^{\circ}$. These crystals had a spectrum with a maximum at $265\text{ m}\mu$ and a antirachitic action almost twice as great as that of the English standard preparation. It is quite possible, that the crystals obtained in so small amount by the English workers contain as chief constituent our substance of melting point $122\text{--}123^{\circ}$; but we cannot say this with certainty, because we have not been able to obtain crystals by the English procedure and consequently a direct comparison was not possible for us.* The English workers have raised the question of whether the crystalline vitamin isolated by them is the sole vitamin D or only one of several vitamins present in the irradiation product.

* Unfortunately I only heard of the new and very important experiments of Dr. Bourdillon and his co-workers after my arrival in London and was therefore unable to discuss their work. As result of improvements in their distillation method the English investigators now obtain a 20 per cent. yield of crystals; these melt at $123\text{--}125^{\circ}$ and have approximately the same spectrum and the same antirachitic action as ours (Angus and others, 1930). Nevertheless the two crystallised substances cannot be identical, for we find even after 10 recrystallisations a rotation of $[\alpha]_{\text{NaCl}}^{18^{\circ}} = 189^{\circ}$, while Bourdillon and his co-workers give the value of $[\alpha]_{\text{NaCl}}^{20^{\circ}} = 280^{\circ}$; this difference cannot be explained by the fact that we used acetone as solvent, whilst Bourdillon used alcohol as solvent.

They incline to the latter view, and I think it very probable that they are right. But I would like to point out, that the vitamin D isolated by us is at least responsible for a great part of the antirachitic action in our products.

In conclusion, an attempt may be made to answer the question : What takes place during the irradiation of ergosterol ?

I believe that, in the photochemical transformation into vitamin D, the molecular formula, the alcohol group and the three double bonds, remain unchanged, and that all that occurs is a steric or structural re-arrangement which leads to an increase in the spatial size of the molecule.

In over-irradiation, on the other hand, the double bonds which in ergosterol and in vitamin D form a conjugated system, are moved further away from each other. As a result, the power to react with maleic anhydride, the property of being reduced with sodium and alcohol, and the characteristic spectrum disappear. It is easy to understand why the photochemical reaction finally leads to sterols without conjugated double bonds, if it is borne in mind that, in the absence of oxygen, these sterols are no longer attacked by ultra-violet rays of wave-lengths longer than 240 m μ , whereas sterols with conjugated double bonds are sensitive to such rays.

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The Colorimetric Properties of the Spectrum.

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Abstract.

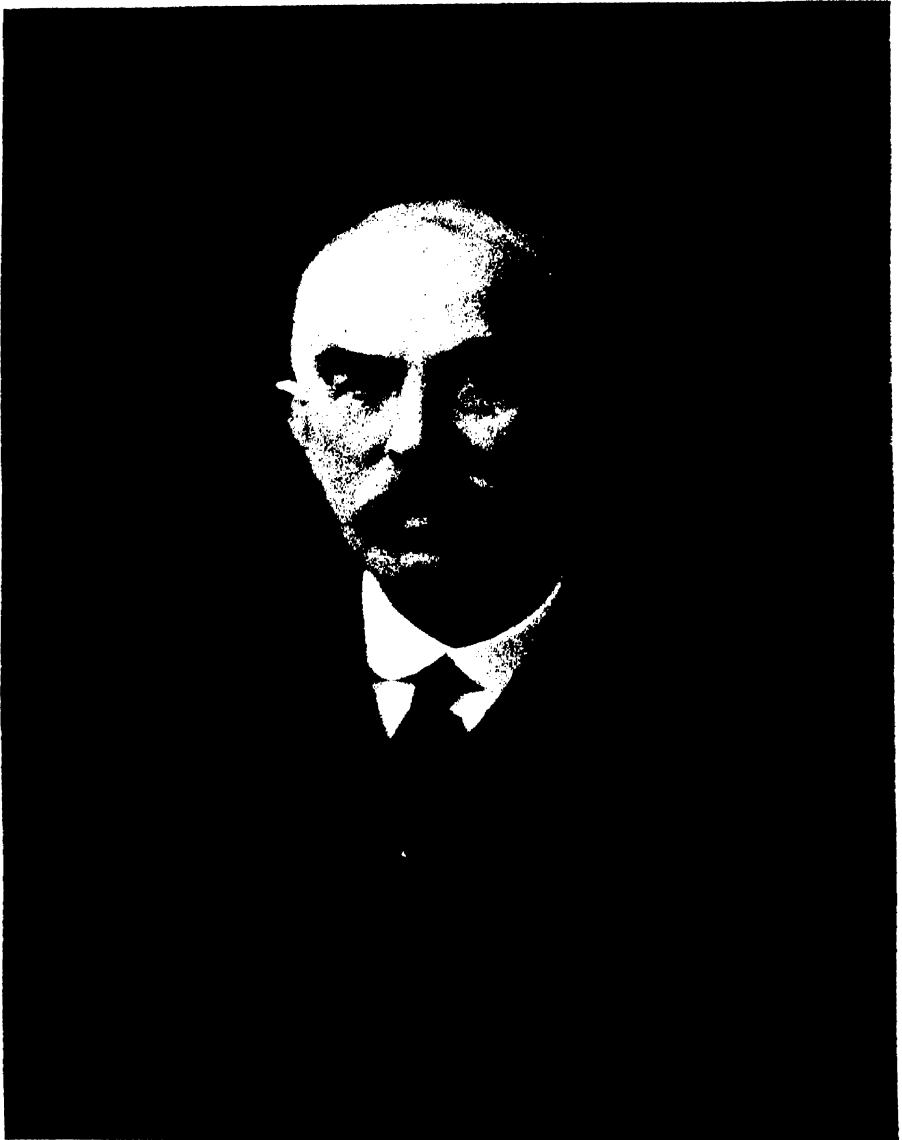
The paper describes an investigation carried out at the National Physical Laboratory to determine the colorimetric properties of a group of seven subjects as obtained from direct measurements of the trichromatic coefficients of the spectrum on a trichromatic colorimeter. The "spectral distribution curves of the primaries," by means of which the colorimetric quality of a heterochromatic stimulus may be computed from its energy distribution curve, are obtained by combining the experimentally determined trichromatic coefficients with the International Standard visibility curve. This procedure is a simplification, applicable to the mean results of a normal group, of a general method by which the chromatic and luminosity functions of any subject or group of subjects can be determined from one set of observations. The general method is described in an Appendix.

The results of the investigation are compared with those obtained by other workers, and a proposal is made for the adoption of a set of standard data, to represent a normal eye for technical colorimetric purposes, based on the results of this investigation and those recently published by W. D. Wright. A complete set of data, worked out on this basis, is included in the paper.

(The full paper is printed in 'Philosophical Transactions,' A, No. 685, which will form pp. 149-187 of vol. 230.)

OBITUARY NOTICE.

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THOMAS BARLOW WOOD.

THOMAS BARLOW WOOD—1869-1929.

For two and twenty years T. B. Wood occupied the Chair of Agriculture at Cambridge. He brought to that office qualifications which were quite exceptional.

A department of agriculture in a University must, to justify its academic status, be permeated with the spirit of research. More, however, than most University departments it must deal with practical problems and minister to practical needs; while in its teaching theory and practice must be nicely balanced. To exert due influence on agricultural progress in its own district its staff must, in an advisory capacity, understand the mentality of the practical farmer and meet with sympathy his sometimes prejudiced views. Finally, if it is to attain to national importance it must, more than other scientific departments, be in touch with administrators, and retain the confidence of Government officials. To control, guide, and represent a school with such diverse activities calls for qualifications seldom combined in an individual, but in a quite remarkable fashion Wood united them all. His training, his experience, his talents and his tastes were all such as to fit him ideally for the position he held, and under him an active school of agriculture grew up at Cambridge, which has served the country well, and of which the University has good reason to be proud.

Wood came of farming stock and throughout his professorial life remained an active farmer, successfully running his Norfolk farm on practical business lines.

He was born in 1869, being the son of E. D. Wood of Field Dalling, Holt, Norfolk. Educated at the Newcastle High School, Staffordshire, he proceeded to Gonville and Caius College, and in 1889 obtained a first class in the Natural Sciences Tripos. In 1908 he was elected to a Fellowship of his College.

In the early nineties national funds became available for technical education, and there was a call for teachers of agriculture and agricultural science. Wood was at that time appointed teacher for the County Councils of Norfolk and Devon. He was then for a time science master at the Norfolk County School. Holding such posts he early acquired an experience in teaching, which, added to a natural gift for clear exposition, later on made professorial lecturing easy for himself and highly profitable to his audiences.

When a School of Agriculture for the Eastern Counties was founded at Cambridge, Wood returned to the University and became secretary to the School. Later he was made Reader in Agricultural Chemistry and held that post till, in 1907, he succeeded to the Chair which eight years earlier had been endowed by the Drapers' Company.

Much of Wood's earlier research work was done in the laboratory, and in the middle nineties he published, alone or conjointly, several papers of chemical

interest of which the most interesting, perhaps, were those (with Spivey and Easterfield) upon the chemical constituents of Indian hemp. The isolation and properties of the essential active principle, Cannabinol ($C_{15}H_{24}O_2$), were first described in these papers.

Wood's interest, however, early turned to the problems of animal nutrition, and these remained always his chief concern as an investigator. He early took a leading part, for instance, in co-operative experiments dealing especially with the winter feeding of bullocks. These were carried out in various Norfolk farms, and were such as to engage the sympathy and help of practical farmers in the district. Other practical questions engaged his attention, however, in the days before he became professor. He contributed to the study of Mendelian inheritance in domestic animals by crossing Dorset Horn and Suffolk sheep, and his study of the resulting F₂ progeny provided valuable information concerning the inheritance of horns and face colour. He was awake to the importance of the statistical treatment of agricultural data, and, in conjunction with Stratton, he published a paper on the growth of mangolds, which was one of the earliest studies of the experimental error involved in field trials. He also applied statistical methods with significant results to a mass of analytical data dealing with the composition of root crops. Just before he was elected to the Chair he had been engaged upon a practical problem of a different kind; one of great importance to the milling and bread-making industries. As is well known, the production of a well-formed, marketable loaf of wheaten bread demands of the flour used that it should be adequate in what has long been known as "strength." A satisfactory definition of this quality and a method for measuring it were lacking; ordinary chemical analysis having failed to yield adequate clues. Wood was conversant with current developments in the physical chemistry of colloids, and by applying this knowledge he was able to throw considerable light on the problem. He described, moreover, a method for the determination of "strength," which can be carried out in a single grain of wheat. In his papers on the subject he made it clear that two factors determine the size and shape of a loaf, namely, the gas-making power of the flour and the elasticity of the gluten, and he defined certain of the factors which control these variables.

When he succeeded to the Chair the School of Agriculture, under the influence of his stimulating personality, rapidly grew in numbers and activity, but its financial resources were not equal to the larger enterprises of agricultural research. Two years later, however, in 1909, the Development Commission was set up, and ultimately under its auspices two primary research institutes were placed at Cambridge—one, for Animal Nutrition under Wood, and another, for Plant Breeding under Biffin. Chemical work upon foodstuffs, digestibility experiments, and the like, were actively pursued in the former and extensive feeding experiments on a large scale were planned. Before these last

were seriously begun the war intervened and made them impossible. After the war Wood became convinced of the importance of applying calorimetry to farm animals on modern lines, and, with the valuable assistance of J. W. Capstick, of Trinity College, an efficient calorimeter was constructed in the School of Agriculture. The earliest work done with this instrument comprised a study of the energy expenditure of the pig during successive stages of its growth. Results were obtained which possess both practical and theoretical interest. At this time Wood endeavoured to find a method for computing rations for all classes of farm animals on the basis of (a) a maintenance-ration related to the animal's live-weight, plus (b) a production-ration estimated in accordance with the amount and kind of product—such as meat, milk, or work—which the animal is meant to produce. He expressed these requirements in the form of a general equation :

$$R = Am + gc,$$

where R is the energy of the ration, A the surface area of the animal, m its maintenance requirements per unit surface area, g its gain in live-weight and c the energy stored in unit live-weight increase. If m and c are known this equation makes it possible to compute the ration required to produce any desired rate of live-weight. But Wood pointed out that the same equation may be used to solve other problems. Suppose, for instance, that R is known but m and c unknown. Then if we have two equations, which can be solved as ordinary simultaneous equations, it is possible to find the values of m and c . Such simultaneous equations, however, have value only when based upon numerous data obtained under circumstances as varied as possible. It would take too long to explain the final development of this method, which was successfully tested in practice. It involved no new principles, but it enabled the problems of rationing to be very clearly visualised, and it is mentioned here as illustrating the trend of Wood's endeavours in his latest years. Among other problems which were interesting him towards the end was a study, ably carried out by H. W. Woodman, but inspired by himself, of the nutritional values of pasture. Almost his last work, published with W. S. Mansfield, dealt with the maintenance and production requirement of ewes and lambs ; this involved a research quite exceptional in its thoroughness and accuracy.

Wood's services during the war were conspicuous. On the Food Council, on the Interallied Food Commission, on the Royal Society Food Committee, and on other bodies dealing with the national food supply, his wide practical knowledge proved invaluable, and scarcely less valuable at such a time was the cheery optimism which characterised his outlook.

In his University Department Wood won admiration and affection from colleagues and pupils alike. He was always accessible, and when consulted never spared time or trouble in affording help. To his intimates he was a charming companion displaying wide interests and sympathies, and an engaging brand of humour which was entirely his own.

F. G. H.



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